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Integrated renal and neural mechanisms contributing to sodium homeostasis and blood pressure regulation

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Dissertation

**INTEGRATED RENAL AND NEURAL MECHANISMS CONTRIBUTING TO
SODIUM HOMEOSTASIS AND BLOOD PRESSURE REGULATION**

by

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Submitted in partial fulfillment of the
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*When I look at you, and you look at me,
I wonder what wonderful things you will be.*

- Emilie Winfield Martin

DEDICATION

To Audrey and Mira, who made this all so much better.

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**INTEGRATED RENAL AND NEURAL MECHANISMS CONTRIBUTING
TO SODIUM HOMEOSTASIS AND BLOOD PRESSURE REGULATION**

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ABSTRACT

Hypertension affects one in two adults in the United States and contributes to more than 10% of deaths worldwide. The salt sensitivity of blood pressure, a clinical phenomenon present in one half of hypertensive patients and one quarter of normotensive individuals, predicts the development of hypertension. The prevalence of hypertension rises with age, and age-related increases in salt sensitivity and sympathetic nervous system activity, which promotes renal sodium reabsorption and plays a pathophysiological role in salt sensitivity and hypertension, have been documented. Increased mechanistic insight into the integrated renal and neural mechanisms influencing sodium homeostasis and blood pressure, particularly in aging, could yield valuable information for the phenotypically targeted treatment of hypertension.

The renal nerves, comprised of the sensory afferent renal nerves (ARN) and the efferent renal sympathetic nerves, influence sodium homeostasis and blood

pressure. The ARN, which include mechanosensitive and chemosensitive fibers, mediate a sympathoinhibitory reno-renal reflex that suppresses renal sympathetic nerve activity. The renal sympathetic nerves release norepinephrine, which can promote salt-sensitive hypertension in part by activating the sodium chloride cotransporter (NCC).

In this thesis, Sprague Dawley rats were used as a model of normal aging to demonstrate that 1) the ARN are critical to the sympathoinhibitory and natriuretic responses to alterations in sodium homeostasis and protect against salt sensitivity of blood pressure, 2) the paraventricular nucleus of the hypothalamus may be a site of central integration of the mechanosensitive sympathoinhibitory reno-renal reflex, 3) norepinephrine promotes NCC activity through an α_1 -adrenoceptor-gated WNK1-OxSR1-dependent signaling pathway, driving salt-sensitive hypertension, and 4) impairments in the sympathoinhibitory reno-renal reflex may promote sympathoexcitation and NCC-mediated sodium retention, driving salt-sensitive hypertension in aging rats. Finally, data from the Genetic Epidemiology of Salt Sensitivity study were used to demonstrate that variance in the gene encoding *Gai2* proteins, which are upregulated in the paraventricular nucleus during high salt intake in salt-resistant animal models and are required for dietary sodium-evoked suppression of renal sympathetic outflow, may be a biomarker for the salt sensitivity of blood pressure in humans. Together, these findings highlight the integrated renal and neural mechanisms contributing to salt sensitivity and age-related hypertension.

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LIST OF ABBREVIATIONS

ADNX	Selective afferent renal nerve ablation
ANOVA.....	Analysis of variance
ARN.....	Afferent renal nerves
CGRP	Calcitonin gene-related peptide
C _{H2O}	Free water clearance
DOCA	Deoxycorticosterone acetate
DSR.....	Dahl Salt Resistant
DSS	Dahl Salt Sensitive
ELISA	Enzyme-linked immunosorbent assay
ENaC.....	Epithelial sodium channel
GenSalt	Genetic Epidemiology of Salt Sensitivity
GFR.....	Glomerular filtration rate
HCTZ.....	Hydrochlorothiazide
HR	Heart rate
HS	High salt
ISO	Isoproterenol
KIM-1	Kidney injury molecule-1
KS-WNK1	Kidney-specific isoform of WNK1
L-WNK1	Full length isoform of WNK1
MAP.....	Mean arterial pressure
NCC.....	Sodium chloride cotransporter

NE	Norepinephrine
NHE3.....	Na ⁺ /H ⁺ exchanger
NKCC2	Na-K-2Cl cotransporter
NS	Normal salt
OxSR1	Oxidative stress responsive kinase 1
PE.....	Phenylephrine
PRA	Plasma renin activity
PVN.....	Paraventricular nucleus
RBF	Renal blood flow
RPP	Renal pelvic pressure
SD	Sprague Dawley
SNP	Single nucleotide polymorphism
SPAK.....	STE20/SPS1-related proline-alanine-rich kinase
TRPV1	Transient receptor potential cation channel subfamily V member 1
UAGT	Urinary angiotensinogen
UNaV	Urinary sodium excretion rate
V	Urinary flow rate
VE.....	Volume expansion
WNK.....	With no lysine kinase

CHAPTER ONE: Introduction

Hypertension

Hypertension, or high blood pressure, affects approximately one in two adults above the age of 18 in the United States (Muntner et al., 2018; Whelton et al., 2018). As the strongest risk factor for stroke, myocardial infarction, and chronic kidney disease, hypertension contributes to more than 10% of deaths worldwide and represents a significant public health issue that is projected to be the leading cause of death and disability by the year 2020 (Kannel, 2000; Lifton et al., 2001; Danaei et al., 2011; Statistics, 2011; World Health, 2013). Normal blood pressure is defined as a systolic blood pressure of less than 120mmHg and diastolic blood pressure of less than 80mmHg, and the importance of small increases in blood pressure is highlighted by a recent change in United States guidelines that reduced the threshold for diagnosis of hypertension from 140mmHg systolic or 90mmHg diastolic to 130mmHg systolic or 80mmHg diastolic blood pressure (Whelton et al., 2018). There is a well-documented benefit of blood pressure reduction on hypertension-related morbidity and mortality (Rashid et al., 2003; Group et al., 2015; Hardy et al., 2015). However, therapeutic control of blood pressure to the recommended target of less than 130/80mmHg is achieved in less than one half of hypertensive patients (Muntner et al., 2018).

Effective blood pressure control remains elusive in part because the mechanisms that promote hypertension are incompletely understood. Approximately 90-95% of hypertensive patients have essential hypertension, in

which no secondary cause of increased blood pressure can be identified (Carretero & Oparil, 2000). In these individuals, hypertension represents a heterogeneous disease that may be driven by impairments in one or more of the physiological systems contributing to blood pressure regulation, which include the vascular, neurohumoral, and renal systems (Oparil et al., 2003). Currently, anti-hypertensive medications targeting each of these systems are prescribed broadly and with little regard to individual phenotype, and anti-hypertensive drug development has been virtually stagnant for more than a decade (Table 1.1) (Shah & Stafford, 2017). There is a critical need for new insight into the mechanistic underpinnings of essential hypertension, which will aid in the identification of new therapeutic targets and guide the phenotypically targeted use of existing interventions.

Primary Target	Class/Mechanism
Renal System	Thiazide diuretics (NCC antagonists)
	Loop diuretics (NKCC2 antagonists)
	Potassium sparing diuretics (aldosterone antagonists)
Sympathetic nervous system	α -blockers
	β -blockers
	Central sympatholytics
Cardiovascular system	Vasodilators
	Calcium channel blockers
Renin angiotensin aldosterone system	Angiotensin-converting enzyme inhibitors
	Antiotension receptor antagonists
	Direct renin inhibitors
	Aldosterone antagonists (potassium sparing diuretics)

Table 1.1. Major anti-hypertensive therapeutics. First line therapies recommended for the general population are indicated in bold type.

Salt Sensitivity of Blood Pressure

The salt sensitivity of blood pressure, defined as an exaggerated pressor response to increased dietary sodium intake, predicts the development and severity of hypertension and increases the risk of its associated adverse clinical outcomes (Weinberger et al., 1986; Weinberger, 1996; Morimoto et al., 1997; Meneton et al., 2005; Franco & Oparil, 2006; Appel et al., 2011). In salt-resistant individuals, dietary sodium-evoked sympathoinhibition facilitates natriuresis and maintenance of normotension (Lohmeier et al., 1999; Brooks et al., 2005; Gao et al., 2005; Osborn et al., 2008; Johns et al., 2011; Johns, 2014). In contrast, salt sensitivity is characterized by a sympathoexcitatory response to dietary sodium intake that promotes sodium retention and hypertension (Bayorh et al., 1998; Miyajima & Yamada, 1999; Huang et al., 2001; Dobesova et al., 2002; Brooks et al., 2005; Yatabe et al., 2010; Stocker et al., 2013; Johns, 2014). While it has been estimated that approximately one half of hypertensive individuals and one quarter of normotensive individuals exhibit salt sensitivity of blood pressure, clinical tests to determine salt sensitivity versus salt resistance are poorly standardized, cumbersome, and rarely used (Weinberger et al., 1986). Salt sensitivity is particularly relevant given that the average daily sodium intake in the United States is roughly 3.5g per day and far exceeds the recommendations of the American Heart Association (1.5g/day) and World Health Organization (2g/day) (Appel et al., 2011; Powles et al., 2013; Mozaffarian et al., 2014). The factors that determine salt sensitivity versus salt resistance of blood pressure are incompletely characterized

but likely include impairments in the sympathetic nervous system regulation and renal sodium handling.

Overview of Blood Pressure Regulation

Blood pressure is determined by cardiac output and total peripheral resistance. Cardiac output is a function of heart rate and stroke volume and reflects the volume of blood pumped through the heart each minute, while total peripheral resistance reflects the resistance to blood flow within the systemic vasculature and is influenced primarily by changes in vessel diameter and blood viscosity. These factors are in turn modulated by the integrated actions of the cardiovascular, neurohumoral, and renal systems (Figure 1.1) (Cowley, 2006).

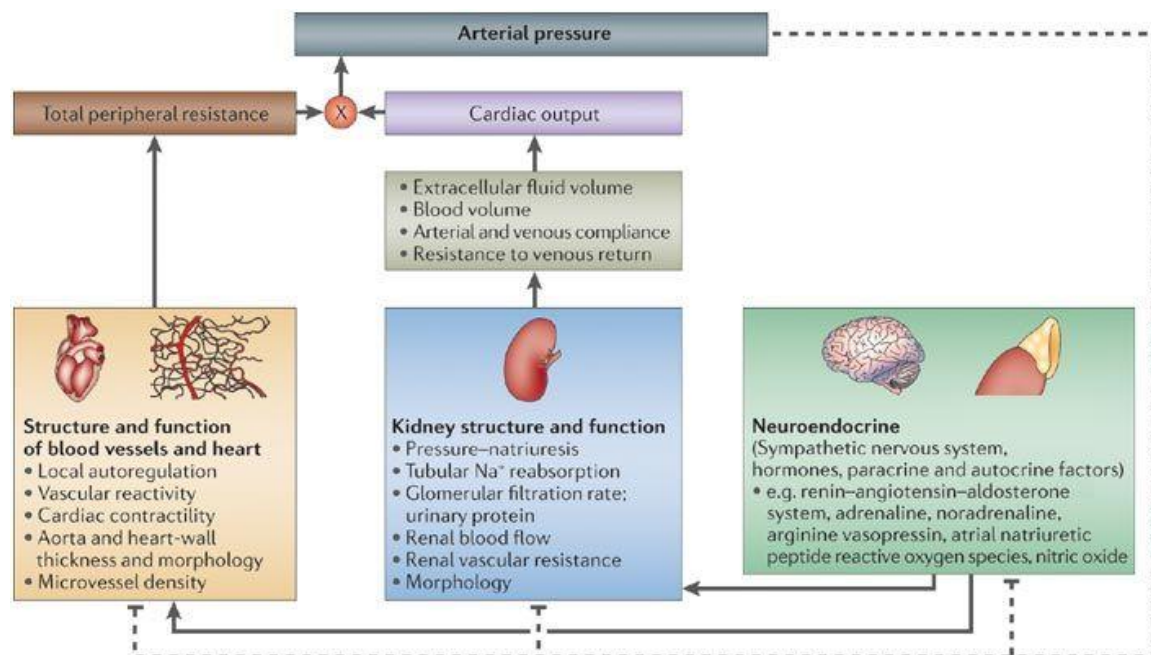


Figure 1.1. An overview of blood pressure regulation. Blood pressure is determined by the cardiovascular, renal, and neurohumoral mechanisms that influence total peripheral resistance and cardiac output. Reproduced from Cowley, *Nature Reviews Genetics* (2006) 7:829-840.

The involvement of both the cardiovascular system and the sympathetic nervous system in blood pressure regulation is highlighted by baroreceptor reflexes that play a dominant role in acute, moment-to-moment blood pressure regulation (Cowley et al., 1973). The baroreceptor reflexes are mediated by stretch-sensitive arterial mechanoreceptors that are activated by acute alterations in blood pressure and in turn influence parasympathetic and sympathetic outflow to the heart and vasculature. Momentary decreases in blood pressure are rapidly corrected via an increase in sympathetic outflow that promotes vasoconstriction, increased cardiac contractility, and increased heart rate as well as a decrease in parasympathetic nerve activity that contributes to increased heart rate. In contrast, acute increases in blood pressure are corrected via decreases in sympathetic outflow and increases in parasympathetic outflow to the heart and vasculature.

In addition to a role in the acute reflex modulation of blood pressure, the sympathetic nervous system plays a role in long-term blood pressure regulation that will be described in greater detail in the **Sympathetic Nervous System and Blood Pressure Regulation** section. In brief, the sympathetic nervous system can influence blood pressure directly through the effects of norepinephrine, which increases heart rate, promotes vasoconstriction, and drives renal sodium reabsorption. Further, norepinephrine released by the renal sympathetic nerves can modulate blood pressure indirectly by stimulating the release of renin and activating the renin angiotensin aldosterone system. The ultimate effectors of the renin angiotensin aldosterone system are angiotensin II, a potent vasoconstrictor

that also stimulates renal sodium reabsorption, sympathetic nervous system activity, and vasopressin secretion, and aldosterone, which promotes renal sodium reabsorption. Notably, each of these systems influences blood pressure in part by altering renal sodium handling, and all anti-hypertensive therapeutics influence renal sodium excretion either directly or through indirect effects on the cardiovascular system, the sympathetic nervous system, or the renin angiotensin aldosterone system (Table 1.1) (Ivy & Bailey, 2014).

Direct evidence for a role of the kidney in blood pressure regulation was first observed during the development of the Goldblatt model of hypertension, in which experimentally induced renal ischemia was found to be sufficient to drive an increase in blood pressure (Goldblatt et al., 1934). Decades later, the Guytonian paradigm, in which the kidneys influence blood pressure via the regulation of extracellular fluid volume, provided mechanistic insight into the relationship between renal sodium handling and blood pressure regulation (Guyton et al., 1972). In this paradigm, blood pressure is maintained by a classical pressure-natriuresis relationship in which an increase in blood pressure drives an increase in renal perfusion pressure, promoting natriuresis and an associated diuresis that reduces extracellular fluid volume and returns blood pressure to normal. Conversely, a decrease in blood pressure results in a decrease in renal perfusion pressure, promoting renal sodium reabsorption and an increase in extracellular fluid volume that increases blood pressure. According to the Guytonian model of blood pressure regulation, hypertension and the salt sensitivity of blood pressure

are characterized by aberrations in the pressure-natriuresis relationship – hypertension by a rightward shift of the pressure-natriuresis curve, and salt sensitivity of blood pressure by a reduction in the slope of the curve – which may reflect pathological changes in renal excretory function and the neurohumoral systems that influence it.

Renal Sodium Handling and Blood Pressure Regulation

Overview of the Kidney

The kidneys are critical to the maintenance of fluid and electrolyte balance, among other critical physiological functions that include waste excretion and acid-base homeostasis. Blood enters the kidney through the afferent renal artery, which is part of a neurovascular bundle that includes the renal vein and mixed sensory afferent and efferent sympathetic renal nerves, and eventually reaches the afferent renal arterioles. Each afferent arteriole empties into a glomerular capillary tuft that is surrounded by Bowman's space, or the luminal space within a renal corpuscle that serves as the start of a nephron. The close proximity of the capillary and the lumen of Bowman's capsule allows for the filtration of fluid and small solutes from the capillaries into the lumen of the nephron, the functional unit of the kidney. The normal glomerular filtration rate, or volume of filtrate entering the lumen per unit time, is 180 L/day (120mL/min) in an average, healthy adult, indicating that the entire 3 liters of plasma in an average adult is filtered into Bowman's capsule approximately 60 times per day. Approximately 25,000 mmol sodium is also filtered

each day, and almost all of the filtered water and sodium must be reabsorbed along the length of the nephron in order to maintain fluid and electrolyte homeostasis.

Each human kidney is composed of approximately 1 million nephrons, and each nephron consists of a series of segments, including Bowman's capsule, the proximal convoluted tubule, the Loop of Henle, the distal convoluted tubule, and the collecting duct, which empties into a calyx of the renal pelvis and then into the ureter (Figure 1.2). The movement of water, sodium, and other solutes between the lumen of the nephron and the bloodstream varies in each of these sequential segments based on the differential expression and activity of luminal and basolateral transporters in the epithelial cells that line the nephron, the external milieu surrounding the nephron, and the physiological needs of the body. In all segments, activity of the basolateral Na^+/K^+ ATPase creates an electrochemical gradient that drives the reabsorption of sodium via luminal transporters.

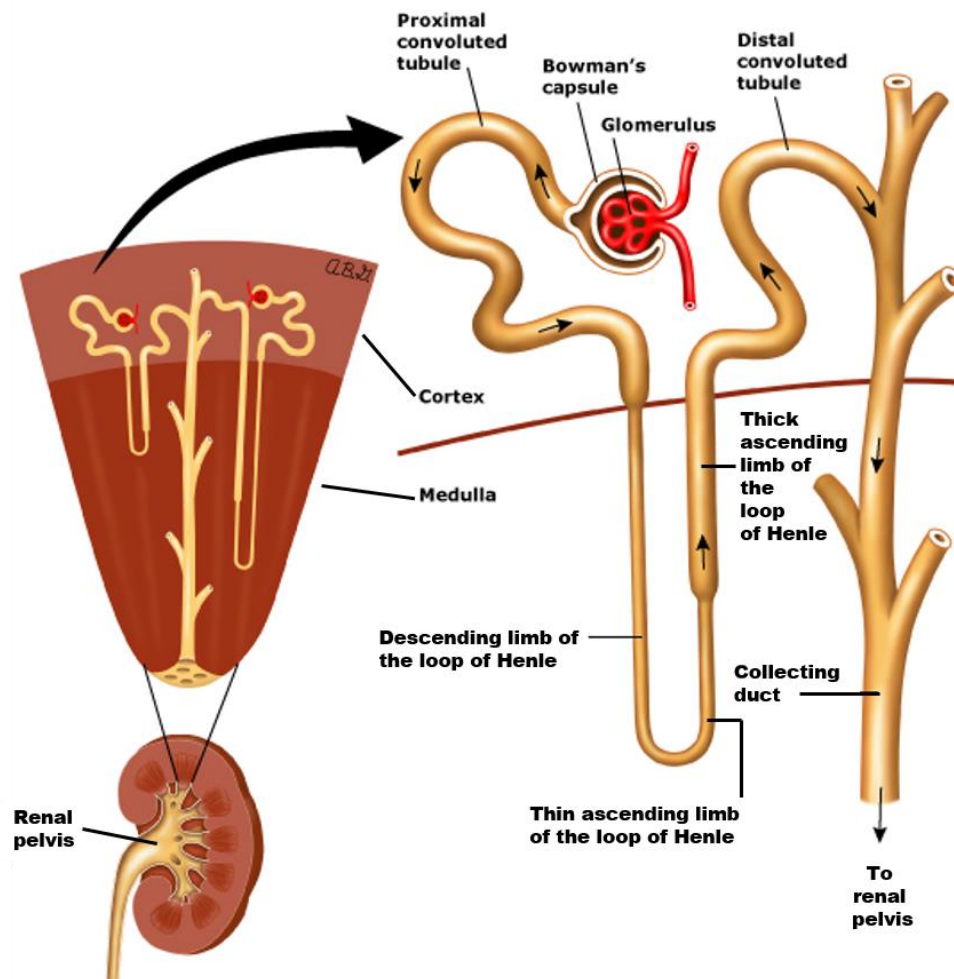


Figure 1.2. Anatomy of the kidney. Adapted from UptoDate (2019).

The Proximal Tubule and the Na^+/H^+ Exchanger

The proximal tubule, situated primarily in the cortex and extending into the outer medulla, is the site of reabsorption of approximately two thirds of filtered water and sodium. In the early part of the proximal tubule, the renal epithelium generates protons in a process that also creates bicarbonate. The luminal Na^+/H^+ exchanger (NHE3) moves protons into the lumen in exchange for sodium, while

the bicarbonate ion moves into the bloodstream. This process is critical to the regulation of both sodium homeostasis and acid-base balance. Sodium is also reabsorbed with glucose, phosphates, and amino acids in the early proximal tubule, and in the late proximal tubule sodium is reabsorbed in a process that essentially equates to cotransport with chloride, although the two ions are transported via different mechanisms. Throughout the proximal tubule, sodium reabsorption drives the proportional reabsorption of water. The bulk reabsorption of sodium and water in the proximal tubule is essential to life, and changes in proximal tubule sodium reabsorption are often compensated for by opposing changes in the distal nephron.

The Loop of Henle and the Na-K-2Cl Cotransporter

The loop of Henle, which descends into the inner medulla and then ascends through the medulla into the cortex, receives a modified fluid from the proximal tubule that is roughly isosmotic compared to plasma. The loop of Henle is comprised of a descending limb, a thin ascending limb present only in a subset of nephrons, and a thick ascending limb, and is responsible for the reabsorption of roughly 25% of filtered sodium and 10% of filtered water. The descending limb of the loop of Henle is permeable to water, but not to sodium or chloride, and water reabsorption is driven primarily by interstitial osmolality, which increases dramatically with depth within the medulla. Selective reabsorption of water creates a more concentrated filtrate, which promotes the passive reabsorption of chloride

through chloride channels expressed in the water-impermeable thin ascending limb. Although the thin ascending limb does not contain sodium transporters, sodium reabsorption passively accompanies chloride reabsorption due to leaky tight junctions between epithelial cells.

All nephrons contain a thick ascending limb, which is also impermeable to water but contains the Na-K-2Cl cotransporter (NKCC2). The NKCC2 uses the sodium gradient created by selective water reabsorption in the descending limb to drive the active reabsorption of one sodium and one potassium ion while removing two chloride ions from the lumen of the nephron. Luminal potassium channels allow the reabsorbed potassium to diffuse back into the nephron such that potassium availability is not a limiting factor for sodium reabsorption. This luminal recycling of potassium results in an imbalance in overall reabsorption whereby the movement of the two chloride ions is matched by only one sodium ion, creating an electrical gradient that drives the paracellular reabsorption of other cations – including sodium.

The Distal Convulated Tubule and the Sodium Chloride Cotransporter

The distal convoluted tubule, which is contained within the renal cortex, receives a modified tubular fluid that is more dilute than plasma due to the net reabsorption of more sodium than water in the loop of Henle. The distal convoluted tubule is responsible for the reabsorption of approximately 5% of filtered sodium and is impermeable to water, while expression of the luminal sodium chloride

cotransporter (NCC) throughout the distal convoluted tubule permits the reabsorption of sodium with chloride. In the late distal convoluted tubule, sodium is also reabsorbed in part via the epithelial sodium channel (ENaC), which generates an electrical gradient driving the paracellular reabsorption of chloride. Although a relatively small proportion of filtered sodium is reabsorbed in the distal convoluted tubule, the NCC is highly responsive to alterations in sodium and fluid homeostasis and therefore plays a critical role in the fine-tuning of renal sodium excretion (Gamba, 2009; Moes et al., 2014).

The Collecting Duct and the Epithelial Sodium Channel

The collecting duct, which extends from the renal cortex through the medulla to empty into the renal pelvis, receives a further diluted fluid from the distal convoluted tubule. The collecting duct is responsible for the reabsorption of approximately 2-5% of filtered sodium and a variable proportion of filtered water that depends largely on fluid status and electrolyte balance. Similarly to the late distal convoluted tubule, sodium is reabsorbed through the ENaC and chloride follows via paracellular diffusion in the collecting duct. Like the NCC, the ENaC influences the fine-tuning of renal sodium excretion. The fluid that ultimately exits the collecting duct into the renal pelvis is excreted as urine and is not further altered in the renal pelvis or the ureter.

The NCC in Health and Disease

Overview of NCC Regulation

The activity of the NCC depends on its total protein expression, localization to the plasma membrane, and phosphorylation at key threonine and serine residues associated with activation. Highlighting the critical homeostatic role of the NCC, the abundance, plasma membrane localization, and phosphorylation of the NCC decrease during high salt intake and increase during low salt intake (Masilamani et al., 2002; Sandberg et al., 2006; Chiga et al., 2008) and similar changes are seen in response to alterations in potassium intake (Vallon et al., 2009; Frindt & Palmer, 2010; Sorensen et al., 2013; Castaneda-Bueno et al., 2014).

NCC activity is modulated in part by hormonal influences that include aldosterone, angiotensin II, vasopressin, and norepinephrine (Rojas-Vega & Gamba, 2016). Aldosterone, administered exogenously or stimulated by a low sodium diet or loop diuretic infusion, increases both the expression and phosphorylation of the NCC (Kim et al., 1998; Abdallah et al., 2001; Masilamani et al., 2002; Chiga et al., 2008; van der Lubbe et al., 2012). Angiotensin II, which promotes aldosterone secretion, can regulate NCC activity independently of aldosterone by promoting NCC phosphorylation and localization to the plasma membrane (Sandberg et al., 2007; San-Cristobal et al., 2009; Zhao et al., 2009; van der Lubbe et al., 2011). NCC phosphorylation is also stimulated by vasopressin (Ecelbarger et al., 2001; Saritas et al., 2013). Infusion of

norepinephrine, the effector of the sympathetic nervous system, increases NCC expression and phosphorylation in mice (Mu et al., 2011; Terker et al., 2014), and prevents dietary sodium-evoked suppression of NCC activity and expression in rats (Walsh et al., 2016). The signaling pathways that mediate the effects of norepinephrine and other modulators of the NCC are complex and incompletely understood but involve a kinase network that includes with no lysine (WNK) kinases, STE20/SPS1-related Proline-Alanine-rich kinase (SPAK), and oxidative stress responsive kinase 1 (OxSR1) (Figure 1.3) (Subramanya & Ellison, 2014; Hadchouel et al., 2016).

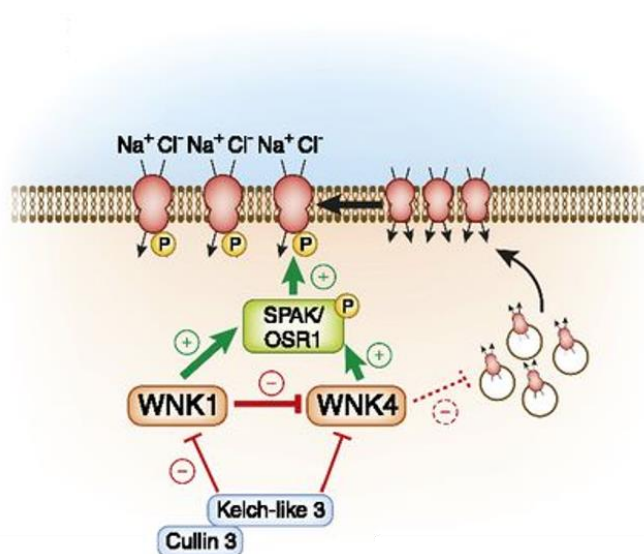


Figure 1.3. Model of the NCC regulatory kinase network. The activity of the NCC depends on its phosphorylation and localization to the plasma membrane. The NCC is phosphorylated and activated directly by SPAK/OxSR1. WNK1 can phosphorylate and activate SPAK/OxSR1 and prevent tonic WNK4-mediated suppression of NCC translocation to the plasma membrane. Kinase-inactive WNK4 prevents the translocation of the NCC to the plasma membrane, while stimulated WNK4 phosphorylates SPAK/OxSR1. Kelch-like 3 and cullin 3 promote degradation of WNK1 and WNK4. Adapted from Subramanya & Ellison, *Clinical Journal of American Society of Nephrology* (2014) 9(12):2147-2163.

The WNK kinases exist as four isoforms, WNK1-WNK4, of which WNK1, WNK3, and WNK4 are expressed in the kidney and have varying effects on the NCC as well as NKCC2 and ENaC (Hadchouel et al., 2016). WNK1 is expressed ubiquitously in its full length isoform, L-WNK1, and in the distal nephron as both L-WNK1 and a kidney specific isoform (KS-WNK1) that lacks kinase activity. WNK1 promotes the phosphorylation and activation of the NCC via a SPAK-dependent pathway in which OxSR1 has also been implicated (Anselmo et al., 2006; Chavez-Canales et al., 2014). KS-WNK1 and a variant of L-WNK1 can also promote NCC activity by inhibiting WNK4, which is expressed in the distal nephron as well as non-renal tissues and reduces renal NCC expression at the plasma membrane in unstimulated conditions by diverting the NCC to lysosomal degradation post-Golgi processing (Wilson et al., 2003; Yang et al., 2003; Yang et al., 2005; Subramanya et al., 2009; Argai et al., 2018). WNK3, present in all segments of the nephron, similarly promotes NCC activity by phosphorylating and inhibiting WNK4, blunting lysosomal degradation and thereby increasing NCC protein expression. Interestingly, while unstimulated WNK4 reduces NCC expression at the plasma membrane, WNK4 also has the capacity to promote NCC phosphorylation and activation in a SPAK-dependent pathway under conditions that include angiotensin II stimulation (San-Cristobal et al., 2009).

SPAK and OxSR1 are serine/threonine kinases that share a nearly 90% homologous kinase domain. WNK1 and WNK4 phosphorylate and activate SPAK and OxSR1, which in turn phosphorylate the NCC at key residues that include

Thr46, Thr55, and Thr60 in the human NCC (corresponding to Thr44, Thr53, and Thr58 in rats and mice) (Moriguchi et al., 2005; Vitari et al., 2005; Richardson et al., 2008). SPAK and OxSR1 have also been implicated in the direct phosphorylation of Ser73 and indirectly in the phosphorylation of Ser91 in the human NCC (Ser71 and Ser89, respectively, in rats and mice) (Richardson et al., 2008; Yang et al., 2010; Filippi et al., 2011). Phosphorylation at Thr60 appears to be particularly important to NCC activation, as a missense mutation that prevents phosphorylation at Thr60 also reduces phosphorylation at Thr44 and Thr53 and significantly reduces NCC activity (Richardson et al., 2008; Glover et al., 2009). Further, phosphorylation at Thr58 in mice (Thr60 in humans) both stabilizes the NCC at the plasma membrane and increases its intrinsic activity (Yang et al., 2013). Demonstrating the relevance of the NCC and its regulatory kinases, mutations in SLC12A3, the gene encoding the NCC, and in the genes encoding WNK4 and WNK1 underlie hereditary disorders characterized by dysregulated renal sodium handling and blood pressure.

The NCC in Monogenic Disorders

Gitelman's syndrome, familial hyperkalemic hypertension, and pseudohypoaldosteronism type II are monogenic diseases characterized by mutations in SLC12A3 and NCC regulatory kinases that ultimately result in altered NCC activity. Homozygous loss-of-function mutations in SLC12A3 lead to Gitelman's syndrome, an autosomal recessive salt-wasting disease in which

hypokalemia, hypomagnesemia, and metabolic alkalosis are accompanied by hypotension (Simon et al., 1996). Interestingly, there is evidence that heterozygous loss-of-function mutations, which do not manifest in an overt clinical syndrome, may actually provide protection against the development of hypertension (Fava et al., 2008; Ji et al., 2008).

In contrast, mutations in the genes encoding WNK1 and WNK4 (Wilson et al., 2001; Vitari et al., 2005; Yang et al., 2007), along with two other regulatory proteins that influence the NCC, kelch-like 3 and cullin 3 (see Figure 3) (Boyden et al., 2012; Louis-Dit-Picard et al., 2012; Glover et al., 2014), cause familial hyperkalemic hypertension, also known as pseudohypoaldosteronism type II. In the case of WNK4, disease-related mutations reduce the inhibitory effect of WNK4 on the NCC and stimulate it to activate SPAK and OxSR1 instead (Yang et al., 2007). In the case of WNK1, intronic mutations drive its overexpression and may thereby drive activation of SPAK/OxSR1-mediated NCC phosphorylation (Wilson et al., 2001). Indicating that NCC-mediated sodium reabsorption is a unifying mechanism driving familial hyperkalemic hypertension, each disease-associated mutation can promote an increase in NCC activity (Wilson et al., 2001; Boyden et al., 2012; Louis-Dit-Picard et al., 2012), and familial hyperkalemic hypertension is exquisitely sensitive to thiazide diuretics targeting the NCC (Mayan et al., 2002).

The NCC in Hypertension

Thiazide diuretics, which compete for the chloride-binding site of the NCC and thereby prevent the cotransport of sodium with chloride, serve as a first-line therapy for essential hypertension (Hughes, 2004). This suggests a potential pathophysiological role for the NCC and its regulatory network in more common, non-genetic forms of hypertension, which has not been directly addressed. Supporting a role for the NCC in hypertension, the blood pressure response to hydrochlorothiazide treatment in hypertensive patients is positively correlated with NCC abundance in urinary exosomes, which reflect total expression levels in the distal convoluted tubule (Pathare et al., 2017). Building upon a report indicating that kidney transplant from normotensive, healthy donors can attenuate resistant hypertension in humans, transplant of a kidney from a patient with Gitelman's syndrome due to a known mutation in SLC12A3 rapidly normalized blood pressure in a patient with kidney failure secondary to longstanding resistant hypertension (Curtis et al., 1983; Stewart et al., 2018) – suggesting that increased NCC activity, corrected by a Gitelman's syndrome transplant with loss-of-function mutations in SLC12A3, may have been driving sodium retention and resistant hypertension.

Angiotensin II has an established role in the regulation of both blood pressure and the NCC, and angiotensin II receptor blockers are approved anti-hypertensive therapeutics. Suggesting a role for the NCC in angiotensin II-evoked hypertension, disruption of the WNK-SPAK signaling pathway attenuates NCC activation and reduces blood pressure in mice treated with angiotensin II

(Cervantes-Perez et al., 2018). The sympathetic nervous system also has an established role in the regulation of blood pressure and NCC activity. While norepinephrine can influence NCC activity directly and indirectly, via activation of the renin angiotensin aldosterone system, several studies have demonstrated that the direct influence of the sympathetic nervous system on NCC activity is particularly relevant to salt-sensitive hypertension (Mu et al., 2011; Terker et al., 2014; Walsh et al., 2016; Liu et al., 2017).

The NCC in Salt-Sensitive Hypertension

Several studies have provided evidence supporting a potential role of the NCC in the pathogenesis of salt-sensitive hypertension (Mu et al., 2011; Terker et al., 2014; Walsh et al., 2016; Liu et al., 2017). Signaling via β_2 -adrenoceptors results in WNK4 downregulation during chronic infusion of norepinephrine or isoproterenol, a non-selective β -agonist, evoking salt-sensitive hypertension associated with an increase in NCC total protein expression, phosphorylation at Ser71 and Thr53, and activity in mice (Mu et al., 2011). Further, salt-resistant Sprague Dawley and Dahl Salt Resistant rats exhibited reduced renal norepinephrine turnover, generally reflective of reduced renal sympathetic outflow, and enhanced WNK4 expression during high salt intake (Mu et al., 2011). In contrast, WNK4 mRNA failed to increase and decreased during high salt intake in DOCA-salt rats and Dahl Salt Sensitive rats, respectively, both of which fail to suppress renal sympathetic tone during high salt intake and exhibit salt-sensitive

hypertension (Mu et al., 2011). Supporting a role for increased NCC activity in the salt-sensitive animals, infusion of isoproterenol during high salt intake promoted NCC activity and sodium retention in salt-sensitive rats (Mu et al., 2011). Another group validated the finding that norepinephrine infusion evokes salt-sensitive hypertension in mice but was unable to reproduce the role of WNK4, calling into question the link between the β_2 -WNK4-NCC signaling pathway and salt-sensitive hypertension (Uchida et al., 2012). However, in another study that used chronic norepinephrine infusion to drive the development of salt-sensitive hypertension in mice, an increase in NCC protein expression and phosphorylation at Thr53 was observed (Terker et al., 2014). The same group used acute infusions of norepinephrine, isoproterenol, and phenylephrine, a specific α_1 -agonist, to investigate NCC regulation in the absence of compensating mechanisms and observed that α_1 - and β -adrenoceptors act synergistically to phosphorylate the NCC via a pathway that requires OxSR1 but not SPAK (Terker et al., 2014). Although the differential roles of β_1 - and β_2 -adrenoceptors were not specifically investigated, β_1 -adrenoceptors were present in distal convoluted tubule cells at far greater density than β_2 -adrenoceptors, leading the authors to hypothesize that β_1 -adrenoceptors may play a more important role in norepinephrine-mediated NCC activation (Terker et al., 2014).

Regardless of the signaling pathways linking norepinephrine with NCC activity, the integrated roles of norepinephrine and the NCC in salt-sensitive hypertension were highlighted in a study demonstrating that norepinephrine

infusion in salt-resistant Sprague Dawley rats evoked salt-sensitive hypertension associated with a failure to suppress NCC activity and expression during high salt intake (Walsh et al., 2016). Critically, chronic infusion of hydrochlorothiazide abolished the salt-sensitive component of norepinephrine-evoked hypertension (Walsh et al., 2016). Together, these observations suggest an important role for the NCC and for the integrated sympathetic nervous system and renal responses to dietary sodium intake in the pathophysiology of salt-sensitive hypertension.

Integrated Renal and Sympathetic Nervous System Regulation of Sodium Balance and Blood Pressure

The sympathetic nervous system influences blood pressure through the effects of norepinephrine in the heart, the systemic vasculature, and kidney. Norepinephrine acts at α_1 -, α_2 -, β_1 -, and β_2 -adrenoceptors that are distributed differentially throughout the body and produce system-specific effects. In the heart, norepinephrine acts primarily at β_1 -adrenoceptors to promote an increase in heart rate, while α_1 -adrenoceptors in the vasculature mediate vasoconstriction. While each of these effects can contribute to an increase in blood pressure, the role of the sympathetic nervous system in long-term blood pressure regulation is derived primarily from its influence on renal sodium handling.

Overview of the Renal Nerves

The renal nerves are comprised of the afferent renal nerves, which carry sensory information from the kidney to the brain, and the efferent renal sympathetic nerves, which convey sympathetic outflow to the kidney. The afferent and efferent renal nerves travel intermingled along the renal artery. Human and animal studies suggest that the efferent renal nerve fibers are far more abundant than afferent renal nerve fibers, with efferent fibers outnumbering afferent fibers by an estimated ratio of approximately 25:1 (Tellez et al., 2013; Sakakura et al., 2014).

The Sensory Afferent Renal Nerves

The afferent renal nerves contain mechanosensitive and chemosensitive fibers that primarily innervate the renal pelvic wall, as well as the renal artery, renal vein, and ureter (Marfurt & Echtenkamp, 1991). The chemosensitive fibers respond to alterations in the chemical composition of urine in the renal pelvis, including increased sodium concentration, and are activated during renal ischemia (Recordati et al., 1980; Recordati et al., 1981). The mechanosensitive fibers are activated by increases in renal pelvic pressure within a physiological range (Smyth et al., 1991; Ma et al., 2002a; Ma et al., 2002b; Kopp et al., 2003) and by the physiological challenge of an intravenous volume expansion, which increases renal pelvic pressure (Genovesi et al., 1993; Chien et al., 1997; Chien et al., 2000).

Afferent renal nerve fibers activated by increased renal pelvic pressure also respond to renal pelvic infusion of substance P (Ma et al., 2002b), a neuropeptide

released from sensory afferent nerve terminals during stimulation. A mechanistic role for substance P in afferent renal nerve activation is further supported by evidence that renal pelvic administration of a substance P antagonist blunts renal pelvic pressure-evoked afferent renal nerve activity (Kopp & Smith, 1993). While the molecular underpinnings of substance P release and subsequent afferent renal nerve activation are incompletely understood, mechanistic studies have implicated bradykinin, prostaglandin E₂, and calcitonin gene-related peptide (Kopp et al., 1997; Gontijo et al., 1999; Kopp et al., 2000).

Afferent renal nerve activation results in physiological responses that are integrated within the central nervous system (Francisco et al., 1980; Kopp et al., 1985). The cell bodies of the afferent renal nerves are contained within the ipsilateral dorsal root ganglia spanning vertebral levels T₆-L₃ (Donovan et al., 1983; Weiss & Chowdhury, 1998), and there is evidence for both monosynaptic projections to the brainstem (Wyss & Donovan, 1984) and polysynaptic signal transmission to sympathetic and cardiovascular regulatory sites within the brainstem and central nervous system that include the rostral ventrolateral medulla, the circumventricular organs, and the paraventricular nucleus of the hypothalamus (Solano-Flores et al., 1997).

The Efferent Renal Sympathetic Nerves

The efferent renal nerves are comprised of postganglionic sympathetic fibers that receive inputs from the brainstem and several sites within the central

nervous system, including the paraventricular nucleus of the hypothalamus, and innervate the renal vasculature, all segments of the nephron, and the renal pelvis. The renal sympathetic nerves synthesize and release norepinephrine, a neurotransmitter which in turn acts upon 1) α_1 -adrenoceptors, promoting renal sodium reabsorption directly via increased activity of sodium transporters including the NCC and indirectly via a decrease in renal blood flow, and 2) β_1 -adrenoceptors, driving renin release and activating the renin angiotensin aldosterone system.

The Reno-Renal Reflexes

Together, the sensory afferent renal nerves and sympathetic efferent renal nerves comprise a reno-renal reflex arc whereby activation of the afferent renal nerves promotes alterations in sympathetic outflow to the contralateral kidney. Two functionally distinct reflexes, one sympathoinhibitory and the other sympathoexcitatory in nature, have been described (Figure 1.4).

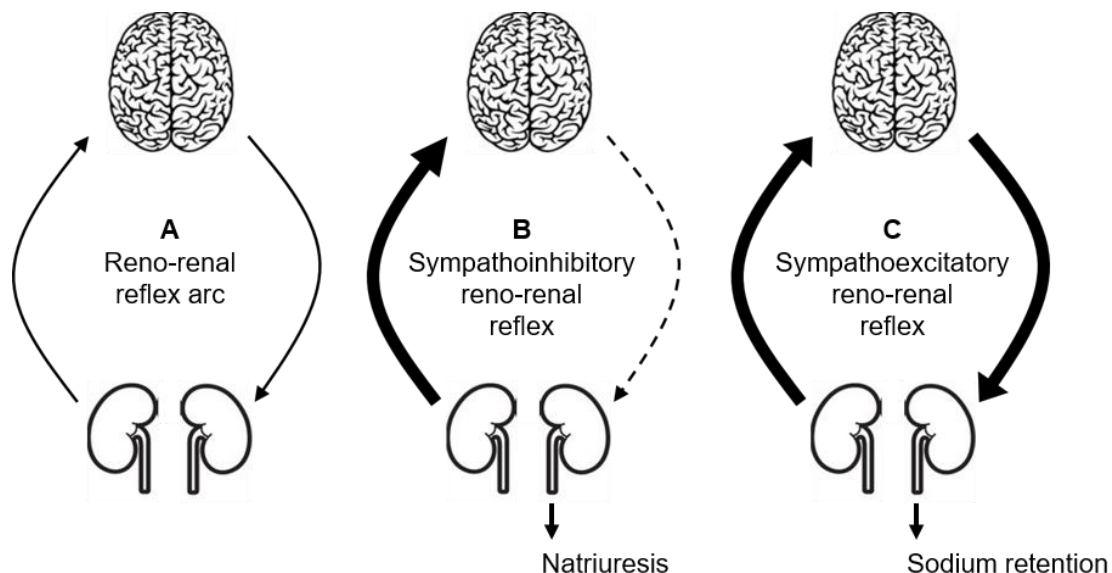


Figure 1.4. Model of the contralateral reno-renal reflexes. (A) Arrows delineate a reno-renal reflex arc composed of the sensory afferent renal nerves originating in one kidney (arrow pointing towards brain) and the renal sympathetic nerves innervating the contralateral kidney (arrow pointing towards kidney), (B) the sympathoinhibitory reno-renal reflex, in which afferent renal nerve activation results in the suppression of sympathetic outflow and a contralateral natriuretic response, and (C) the sympathoexcitatory reno-renal reflex, in which afferent renal nerve activation promotes an increase in sympathetic outflow that drives sodium retention in the contralateral kidney.

Studies in healthy, normotensive animals in which renal pelvic pressure was increased either by direct manipulation (Smyth et al., 1991; Ma et al., 2002a; Ma et al., 2002b; Kopp et al., 2003) or by an intravenous volume expansion (Genovesi et al., 1993; Chien et al., 1997; Chien et al., 2000) have demonstrated that acute activation of the mechanosensitive afferent renal nerves is associated with the suppression of efferent renal sympathetic nerve activity and a natriuretic and diuretic response. These findings indicate that the mechanosensitive afferent renal nerves mediate a sympathoinhibitory reno-renal reflex that facilitates acute sodium homeostasis.

In contrast, activation of an acute sympathoexcitatory reno-renal reflex has been observed following intrarenal infusion of adenosine (Katholi et al., 1983; Katholi et al., 1984) and bradykinin (Barry & Johns, 2015) and during renal ischemia and direct renal pelvic administration of concentrated urine, which activate renal chemoreceptors (Recordati et al., 1982; Rogenes, 1982). The sympathoexcitatory reno-renal reflex promotes sodium retention and has been primarily implicated in disease states that include renal ischemia and heart failure (Zheng et al., 2018).

While afferent renal nerve activity modulates efferent sympathetic outflow to the kidney via the reno-renal reflexes, efferent renal sympathetic nerve activity can also directly influence the afferent renal nerves. Afferent and efferent renal nerve terminals are often found in tight proximity in the renal pelvis, and the efferent renal sympathetic nerves release norepinephrine that can act at α_1 - and α_2 -adrenoceptors to increase and decrease afferent renal nerve activity, respectively (Kopp et al., 2007).

Central Integration of the Reno-Renal Reflexes

Studies using spinal cord transection indicate that the reno-renal reflexes are integrated in the central nervous system (Francisco et al., 1980; Kopp et al., 1985), and direct electrical stimulation of the afferent renal nerves results in Fos induction – a marker of neuronal activation – in a number of brainstem and central nervous system structures that include the paraventricular nucleus of the

hypothalamus, which plays an important role in the modulation of sympathetic nervous system activity, sodium and fluid homeostasis, and blood pressure regulation (Solano-Flores et al., 1997; Guyenet, 2006; Stocker et al., 2010; Zheng & Patel, 2017).

The paraventricular nucleus contains magnocellular and parvocellular divisions that are functionally distinct. The magnocellular division contains neurons that project to the posterior pituitary and release oxytocin or vasopressin into the circulation there (Ferguson et al., 2008). The parvocellular division contains neurosecretory neurons, which modulate the release of hormones including adrenocorticotropin hormone and thyroid-stimulating hormone, and preautonomic neurons, which project to brainstem and spinal autonomic control centers including the nucleus tractus solitarius, rostral ventrolateral medulla, and intermediolateral cell column of the spinal cord to influence sympathetic outflow (Guyenet, 2006; Ferguson et al., 2008; Zheng & Patel, 2017).

A potential role for the paraventricular nucleus in the integration of the renorenal reflexes is suggested by studies in which an acute volume expansion stimulates sympathoinhibitory neurons in the paraventricular nucleus (Haselton et al., 1994; Patel & Zhang, 1994; Randolph et al., 1998; Cunningham et al., 2002; Howe et al., 2004) and drives the suppression of renal sympathetic outflow (Haselton et al., 1994; Kapusta et al., 2012). Further, direct electrical stimulation of the afferent renal nerves activates RVLM-projecting neurons in the paraventricular nucleus; however, the study suggested that activation of these

neurons contributes to increased sympathetic outflow, indicating a potential role for the paraventricular nucleus in the sympathoexcitatory reno-renal reflex (Xu et al., 2015).

There is evidence that the paraventricular nucleus also plays a role in hypertension and salt sensitivity of blood pressure. In salt-resistant animal models, the PVN plays a role in sympathoinhibition, natriuresis, and maintenance of normotension (Sly et al., 2001; Akine et al., 2003; Badoer et al., 2003; O'Donoghue & Brooks, 2006; Frithiof et al., 2014; Larson et al., 2015). In contrast, the PVN evokes sympathoexcitation – due in part to impaired sympathoinhibition – accompanied by sodium retention and hypertension in salt-sensitive animal models (Budzikowski et al., 1998; Huang & Leenen, 1998; Huang et al., 1998; Huang et al., 2001; Dampney et al., 2005; Li & Pan, 2006; Shi et al., 2007; Gabor & Leenen, 2009, 2012; Kim et al., 2013; Bardgett et al., 2014; Choe et al., 2015; Holbein & Toney, 2015). While the mechanistic role of the PVN in the reno-renal reflexes and long-term blood pressure regulation is incompletely understood, several studies have indicated a potential role for central nervous system and PVN-specific $G_{\alpha i2}$ proteins in the determination of salt sensitivity versus salt resistance of blood pressure (Kapusta et al., 2012; Kapusta et al., 2013; Wainford et al., 2015).

Central Gai₂ Proteins in Sodium Homeostasis

The Gai₂ subunit protein is one of several subtypes of α -subunits that serve as effector molecules for G-proteins. In general, G-proteins are composed of α -, β - and γ -subunits and associated with G-protein coupled receptors. Ligand binding at the receptor ultimately results in the dissociation of a linked $\beta\gamma$ complex from the α -subunit, which then initiates a distinct signaling pathway that depends on the subtype of the α -subunit. A number of studies indicate that Gai₂ signaling pathways in the central nervous system are involved in the homeostatic responses to acute and chronic sodium challenges.

In salt-resistant Sprague Dawley, Dahl Salt Resistant, and Brown Norway rats, high salt intake promotes a PVN-specific increase in Gai₂ protein expression (Kapusta et al., 2012; Kapusta et al., 2013; Wainford et al., 2015). In contrast, Dahl Salt Sensitive rats fail to upregulate PVN Gai₂ protein expression during high salt intake (Wainford et al., 2015). Importantly, experimental downregulation of central Gai₂ proteins to prevent sodium-evoked increases in PVN Gai₂ protein expression resulted in sympathoexcitation, sodium retention, and the development of salt-sensitive hypertension during high salt intake in salt-resistant strains (Kapusta et al., 2013; Wainford et al., 2015). Dietary sodium-evoked sympathoexcitation and salt-sensitive hypertension were prevented in normally salt-resistant rats that underwent renal denervation prior to manipulation of central Gai₂ proteins (Kapusta et al., 2013; Wainford et al., 2015).

Several studies have also demonstrated that central Gai₂ proteins are required for renal sympathetic nerve-mediated homeostatic responses to an acute volume expansion and a non-pressor acute hypertonic saline infusion (Kapusta et al., 2012; Wainford et al., 2013). Critically, experimental downregulation of central Gai₂ during a pressor hypertonic saline challenge resulted in impaired sympathoinhibitory and blood pressure responses accompanied by impaired activation of PVN parvocellular neurons (Carmichael et al., 2016). Although further studies using PVN-specific manipulation of Gai₂ proteins during acute and chronic sodium challenge are required, these findings raise the possibility that PVN Gai₂ proteins are critical to the renal nerve-dependent sympathoinhibitory and natriuretic responses that promote salt resistance during chronic dietary sodium intake.

Bilateral Renal Nerve Ablation

A central role of the renal nerves in hypertension is supported by studies in which bilateral renal nerve ablation lowers blood pressure in multiple hypertensive animal models and in human hypertension (Kline et al., 1980; Iliescu et al., 2006; Esler et al., 2012; Foss et al., 2013; Intapad et al., 2013; Johns & Abdulla, 2013; Kapusta et al., 2013; Henegar et al., 2014; Hering et al., 2014; Krum et al., 2014; Bohm et al., 2015; Pires et al., 2015; Wainford et al., 2015; Fong et al., 2016; Foss et al., 2016; Gao et al., 2016). In humans, and often in larger animal models such as sheep, less invasive radiofrequency ablation is used to destroy the renal nerves.

In smaller animal models renal denervation is performed using an invasive surgical procedure in which the renal nerves are physically disrupted and then treated with a phenol solution that destroys any remaining intact fibers. Both techniques remove both the afferent renal nerves and the renal sympathetic nerves.

Although human trials of renal nerve ablation have shown largely positive results, a large, prospective, single-blind, randomized, sham-controlled trial termed SYMPPLICITY HTN-3 failed to reproduce the anti-hypertensive effects of renal nerve ablation demonstrated by the smaller SYMPPLICITY HTN-1 & 2 trials (Bhatt et al., 2014). However, procedural inconsistencies and an inability to verify renal nerve ablation may have confounded the results of SYMPPLICITY HTN-3, and subsequent human trials – including DENER-HTN and three sham-controlled trials, SPYRAL HTN OFF-MED, SPYRAL HTN ON-MED, and Radiance-HTN SOLO – continue to highlight the renal nerves as a viable therapeutic target in hypertension (Azizi et al., 2015; Esler, 2015; Zaldivia et al., 2016; Townsend et al., 2017; Kandzari et al., 2018).

While studies of renal denervation suggest that the renal nerves play a pathophysiological role in hypertension, it has historically been difficult to dissect out the specific roles of the afferent renal nerves versus the efferent renal nerves. The potential importance of the afferent renal nerves in the regulation of sodium handling and blood pressure is highlighted by an increase in afferent renal nerve activity during high sodium intake in salt resistant Sprague Dawley rats, and a decrease in afferent renal nerve activity in the Spontaneously Hypertensive Rat

(Kopp et al., 2007; Kopp et al., 2009; Kopp et al., 2011a; Kopp et al., 2011b). Further, general sensory denervation via dorsal rhizotomy or systemic capsaicin treatment, which removes all afferent inputs including the afferent renal nerves, evokes salt-sensitive hypertension in Sprague Dawley rats (Wang et al., 1998; Wang et al., 2001; Kopp et al., 2003).

Selective Afferent Renal Nerve Ablation

The recent development of a novel technique to selectively ablate the afferent renal nerves has provided a powerful tool in the investigation of the differential roles of the afferent renal nerves and the renal sympathetic nerves in sodium homeostasis and blood pressure regulation (Foss et al., 2015). The technique uses direct application of capsaicin to the renal nerves to activate TRPV1 cation channels expressed on the afferent renal nerves, but not the efferent renal nerves, resulting in selective afferent renal nerve ablation (Foss et al., 2015). Selective afferent renal nerve ablation does not alter baseline cardiovascular parameters or cause localized tissue injury or kidney damage assessed both functionally and histologically.

Studies performed using selective afferent renal nerve ablation indicate that the role of the afferent renal nerves in hypertension depends on both the model and the stage of hypertension under investigation. The afferent renal nerves are not involved in angiotensin II hypertension (Foss et al., 2018), for instance, while selective afferent renal nerve ablation reduces blood pressure in DOCA-salt

hypertension (Foss et al., 2015; Banek et al., 2016; Banek et al., 2018), which is characterized by an increase in afferent renal nerve activity (Banek et al., 2016). Although a high sodium diet enhances afferent renal nerve responsiveness and non-selective sensory denervation evokes salt-sensitive hypertension in Sprague Dawley rats, suggesting a role for the afferent renal nerves in the maintenance of sodium homeostasis (Wang et al., 1998; Wang et al., 2001; Kopp et al., 2003; Kopp et al., 2009), ablation of the afferent renal nerves had no impact on the blood pressure responses to stepped increases in dietary sodium intake (Foss et al., 2015). However, dietary sodium manipulations were carried out during a time frame in which functional afferent renal nerve reinnervation could have occurred and compensatory mechanisms could have masked the effects of afferent renal nerve ablation, and as such these studies require further clarification (DiBona & Sawin, 1983; Mulder et al., 2013; Foss et al., 2015). Further, while most mechanistic studies of both the afferent renal nerves and the renal sympathetic nerves have been conducted in young adult animal models, age-related alterations in blood pressure regulation, sympathetic nervous system activity, and renal sodium handling raise the possibility that the renal nerves may be particularly important in the context of age-related hypertension.

Hypertension in the Aging Population

The prevalence of hypertension rises from roughly one in three adults aged 20-44 to more than three in four adults above the age of 65 (Muntner et al., 2018;

Whelton et al., 2018). Importantly, while aging is associated with increased risk of hypertension-associated morbidity and mortality, the proportion of patients able to achieve therapeutic blood pressure control declines with age (Writing Group et al., 2016; Muntner et al., 2018; Whelton et al., 2018). Although the mechanisms driving age-related hypertension are incompletely understood, aging is associated with an increase in the prevalence of the salt sensitivity of blood pressure, and there is evidence that sympathetic tone is elevated and renal sodium handling is altered in aging animal models and elderly humans (Bengele et al., 1981a; Bengele et al., 1981b; Luft et al., 1987; Luft et al., 1991; Fish et al., 1994).

Modeling Age-Related Hypertension

Despite the marked increase in the prevalence of hypertension and the increased risk of hypertension-associated morbidity and mortality in human aging, relatively few human studies have focused on the mechanisms driving hypertension and salt sensitivity of blood pressure in the specific context of aging. Human studies often set an upper age limit for recruitment due to safety concerns or the presence of comorbidities that could confound the interpretation of the study (Buford, 2016). Conversely, studies in which elderly hypertensive patients are specifically recruited often exclude younger and normotensive individuals, instead focusing on comparisons of treatment groups within the aging, hypertensive study population (Beckett et al., 2008). Human studies of salt sensitivity of blood pressure in aging are also uncommon, likely due to the cumbersome and poorly

standardized methods of defining salt sensitivity that limit these investigations irrespective of aging.

Animal studies of age-related hypertension and salt sensitivity are similarly scarce, perhaps due in part to the ready availability of genetic and experimentally induced models of hypertension and the paucity of models of aging that appropriately mimic age-related alterations observed in humans (Leong et al., 2015). Some animal models of aging are resistant to hypertension and other pathophysiological changes, while others develop hypertension but also exhibit functional renal damage that renders the isolation of aging as a potential casual factor quite difficult (Coleman et al., 1977; Owen & Heywood, 1986; Erdely et al., 2003; Bilusic et al., 2008). In this section, the available human and animal studies of age-related changes that could promote hypertension and salt sensitivity are summarized, with particular focus on the sympathetic nervous system and renal sodium handling.

The Sympathetic Nervous System in Age-Related Hypertension

An age-related increase in sympathetic nervous system activity has been observed in studies using a number of methods, including the assessment of plasma norepinephrine concentration and plasma norepinephrine spillover (Esler et al., 1995; Esler et al., 2002). A direct positive relationship between sympathetic tone and blood pressure is observed only in adults above the age of 40, suggesting

that increased sympathetic tone may be particularly relevant to age-related hypertension (Narkiewicz et al., 2005).

Despite this evidence, relatively few studies have investigated the impact of aging on renal sympathetic outflow. While one study failed to demonstrate an age-related increase in renal norepinephrine spillover in human patients, other studies have demonstrated that human aging is characterized by inappropriate renal artery vasoconstriction, mediated by the renal sympathetic nerves in response to acute physiological challenges, suggesting that renal sympathetic nerve responsiveness may be enhanced in aging (Esler et al., 1995; Kuipers et al., 2009; Patel et al., 2013). Further, electrophysiological studies have provided evidence that aged beagles exhibit increased renal sympathetic nerve activity at baseline and impaired suppression of renal sympathetic nerve activity following an acute volume expansion (Hajduczuk et al., 1991a; Hajduczuk et al., 1991b). Demonstrating a potential mechanistic link between increased renal sympathetic tone and hypertension in aged animals, an increase in renal norepinephrine content is associated with renal sodium retention and elevated blood pressure in aging Wistar-Kyoto rats and spontaneously hypertensive rats (Pinto et al., 2011). Importantly, a recent clinical trial verified that renal denervation effectively reduces blood pressure in elderly hypertensive patients (Ziegler et al., 2015).

Renal Sodium Handling and the NCC in Age-Related Hypertension

Human aging is characterized by a reduction in the ability to maximally concentrate and dilute urine that may promote impaired homeostatic responses to acute and chronic sodium challenge (Schlanger et al., 2010). Increasing age is associated with blunted and delayed natriuretic responses to acute saline infusion, an effect that persists after adjustment for renal damage (Luft et al., 1980; Luft et al., 1982; Fish et al., 1994). Conversely, aging individuals exhibit a delayed return to sodium balance following the initiation of a low sodium diet (Schlanger et al., 2010). While aging is associated with a number of changes in the vascular, neurohumoral, and renal systems that could contribute to sodium retention and hypertension (Frame & Wainford, 2018), age-related increases in sympathetic tone and salt sensitivity and the established interaction of sympathetic nervous system activity and dietary sodium intake in the regulation of the NCC raise the possibility that the NCC is involved in age-related hypertension.

Studies in animal models of aging have yielded conflicting results regarding a potential role of the NCC in age-related hypertension. Aging Fischer Brown Norway rats exhibit increased baseline NCC expression and fail to increase NCC expression in response to water deprivation, and separate studies have demonstrated that these animals develop age-related hypertension (Tian et al., 2006; Chugh et al., 2012; Chugh et al., 2013; Pokkunuri et al., 2015). However, NCC activity was not assessed and evidence from another model of aging, the Sabra rat, suggests that the activity of the Na⁺/K⁺-ATPase decreases with age,

indicating that the driving force for NCC activity is reduced (Scherzer et al., 2015). Importantly, while aged C57Bl6 / CBA / 129 mice exhibit decreased baseline NCC activity, the observation that angiotensin II-evoked increases in NCC activity and blood pressure are exacerbated in aged mice suggests that impaired NCC regulation could promote sodium retention and hypertension regardless of baseline NCC activity (Tiwari et al., 2009). Further, increased age was associated with an enhanced blood pressure response to thiazide diuretics in a small study of hypertensive humans, supporting a potential pathological role of NCC activity in age-related hypertension (Sabanathan et al., 1987).

Hypothesis and Specific Aims

Hypothesis

An age-related impairment in the afferent renal nerve sympathoinhibitory renorenal reflex contributes to sympathoexcitation, leading to sodium chloride cotransporter-mediated sodium retention and age-related hypertension.

Specific Aims

Specific Aim 1: To establish that an age-related reduction in afferent renal nerve activity contributes to impaired neural and renal responses to acute challenges to fluid and sodium homeostasis in Sprague Dawley rats.

Specific Aim 2: To establish that an age-related reduction in afferent renal nerve activity contributes to sodium retention and the development of hypertension in Sprague Dawley rats.

CHAPTER TWO: General Methods

This chapter contains a detailed description of the surgical procedures, in vivo and ex vivo protocols, and molecular and analytical techniques used in studies described in Chapters 3-5. In turn, Chapters 3-5 each contain a brief, chapter-specific methods section with a schematic overview referring to the methods detailed below.

Animals

Male Sprague Dawley, Dahl Salt Resistant, and Dahl Salt Sensitive rats weighing 275-300g (approximately 3 months old) and male Sprague Dawley rats aged 8 and 16 months were purchased from Envigo (Indianapolis, IN, USA). Rats were pair-housed until surgical intervention or body weight required individual housing. All rats were maintained in a temperature-controlled (68-79°F) and humidity-controlled (30-70%) facility under a 12-hour light-dark cycle. Tap water and standard rodent diet or experimental high sodium diet (Table 2.1) were provided ad libitum. In all studies, rats were randomly assigned to experimental groups. All animal protocols were approved by the Institutional Animal Care and Use Committee in accordance with the guidelines of the Boston University School of Medicine and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

	Normal salt	High salt
Envigo Teklad ID	Global diet	TD.03095
Total NaCl content	0.6%	4%
	102 mEq/kg	678mEq/kg
Protein	18%	19%
Crude fat	5%	5%
Fiber	5%	3%

Table 2.1. Nutritional content of normal salt diet and high salt diet obtained from Envigo Teklad.

Surgical Procedures

Osmotic mini-pump implantation

Rats were anesthetized with sodium methohexital (20mg/kg administered intraperitoneally). An incision was made on the ventral neck and a subcutaneous pocket was gently opened in the subscapular region. An osmotic mini-pump with a flow rate of 5 μ L/hour (Model 2ML2, Alzet, CA) containing an experimental infusion (Table 2.2) was inserted and the surgical incision site was sutured closed. Rats received penicillin (300,000 units/kg, administered intramuscularly) and were returned to their home cages.

The 2ML2 pump model, which is designed for a 14-day infusion but offers the highest available flow rate (5 μ L/hour), was used in all studies due to limitations of drug solubility that prevented the use of pumps designed to deliver longer infusions at a smaller flow rate. For studies longer than 14 days, the pump was replaced on day 14.

Infusion	Dose	Reference
Isotonic saline	N/A	
Norepinephrine	600ng/min	Sonalker et al., 2008 Walsh et al., 2016
Terazosin	10mg/kg/day	Maranon et al., 2015
Propranolol	10mg/kg/day	Maranon et al., 2015
Norepinephrine + Terazosin	600ng/min 10mg/kg/day	
Norepinephrine + Propranolol	600ng/min 10mg/kg/day	
Hydrochlorothiazide	4mg/kg/day	Ashek et al., 2012
Losartan	3mg/kg/day	Walsh et al., 2016

Table 2.2. Doses of pharmacological agents used in osmotic minipumps implanted subcutaneously.

Acute femoral vein, femoral artery, and bladder cannulation

On the day of acute study, rats were anesthetized with sodium methohexital (20mg/kg administered intraperitoneally, supplemented with 10mg/kg administered intravenously as required) and cannulation of the femoral vein, femoral artery, and bladder was performed as previously described (Wainford et al., 2013; Carmichael et al., 2016; Walsh et al., 2016). An incision was made in the left femoral triangle and the femoral artery and femoral vein were carefully separated from surrounding tissue. Cannulas made of PE-50 tubing were inserted into the left femoral vein, allowing for the maintenance of intravenous anesthesia during the remainder of the acute surgery and isotonic saline, experimental sodium challenges, or pharmacological agents following recovery, and in the left femoral artery, allowing for the measurement of mean arterial pressure and heart rate and the collection of arterial blood samples in some studies. The surgical incision site

was then sutured closed, leaving the free ends of the vein and artery cannulas externalized. A second incision was made in the pelvic region in order to place a cannula made of PE-240 tubing directly into the bladder to allow for the collection of urine. The surgical incision site was then sutured, again leaving the free end of the cannula externalized. Rats were placed in a Plexiglas rat holder and the femoral vein and artery cannulas were connected to an infusion pump and an external pressure transducer, respectively. Rats underwent a 2-hour surgical recovery period during which an intravenous infusion of isotonic saline (20 μ L/min) was maintained and rats returned to full consciousness and stable renal and cardiovascular function. Mean arterial pressure and heart rate were recorded continuously via the femoral artery cannula using computer-driven data acquisition software (MP150 and AcqKnowledge 3.8.2, BIOPAC, CA) connected to the external pressure transducer (P23XL, Viggo Spectramed Inc, CA).

Renal artery cannulation

In a subset of animals, intravenous sodium methohexital anesthesia was maintained after acute cannulation of the femoral vein, femoral artery, and bladder to allow for cannulation of a renal arteriole before the surgical recovery period. In these animals, a flank incision was made and the distal end of the left renal artery and its arterioles were carefully separated from surrounding tissue. A cannula made of PE-10 tubing was inserted into a branch of the renal artery, allowing for renal infusion of bradykinin (Foss et al., 2015).

Renal pelvis cannulation

Rats were anesthetized with sodium thiopental (20mg/kg administered intraperitoneally). The femoral vein was cannulated as described above in the *Acute femoral vein, femoral artery, and bladder cannulation* section in order to maintain sodium thiopental anesthesia for the duration of the experiment (0.04 mmol/kg/hour at a rate of 50 μ L/min in isotonic saline administered intravenously). An incision was made in the left lateral abdomen and the left ureter was carefully separated from surrounding tissue. A cannula made of PE-50 tubing containing three heat-pulled tips of PE-10 tubing was inserted into the left renal pelvis via the left ureter to allow 1) renal pelvic perfusion and manipulation of renal pelvic pressure, 2) drainage of effluent, and 3) recording of renal pelvic pressure (Kopp et al., 1994; Lin et al., 2015). The surgical incision site was closed, leaving the free end of the cannula externalized. The right ureter was then cannulated via a second incision site using the same techniques but with simple PE-50 tubing to allow for contralateral urine collection. After the second incision site was sutured closed, anesthesia was maintained during a 90-minute rest period that allowed collection of stable steady-state urine followed by an experimental period described in the *Manipulation of renal pelvic pressure* or *Manipulation of renal pelvic sodium concentration* sections of this chapter. Renal pelvic pressure was recorded continuously via the left renal pelvic cannula using computer-driven data acquisition software (MP150 and AcqKnowledge 3.8.2, BIOPAC, CA) connected to the external pressure transducer (P23XL, Viggo Spectramed Inc, CA).

Radiotelemetry probe implantation

Rats were anesthetized with ketamine combined with xylazine (30mg/kg ketamine with 3mg/kg xylazine, administered intraperitoneally) and a radiotelemetry probe (PA-C40, DSI, MN) was implanted as previously described (Brouwers et al., 2015; Foss et al., 2015; Wainford et al., 2015). An incision was made in the left femoral triangle and the femoral artery was carefully separated from surrounding tissue. The catheter of a radiotelemetry probe was inserted into the femoral artery and a subcutaneous flank pocket was created to house the body of the radiotelemetry probe. Rats received penicillin (300,000 units/kg, administered intramuscularly) and were returned to their home cages. In all radiotelemetry studies, rats were allowed to recover for 5-7 days prior to the start of baseline experimental data collection. During experimentation, rats remained in their home cages placed on top of receiver plates. Blood pressure and heart rate data were collected, stored, and analyzed using Dataquest A.R.T. 4.2 software (DSI, MN).

Total Bilateral Renal Denervation, Selective Afferent Renal

Nerve Ablation, and Sham Denervation

Rats were anesthetized with sodium thiopental (20mg/kg administered intraperitoneally). A dorsal flank incision was made and the renal artery and vein were carefully separated from surrounding tissue. Rats underwent one of three manipulations of the renal nerves that run along the renal artery: 1) bilateral renal

denervation, in which visible nerve bundles were physically disrupted and then coated with a 10% phenol solution in ethanol to destroy remaining nerve fibers, 2) selective afferent renal nerve ablation, in which the nerves were not physically disrupted but a capsaicin solution (33mM in isotonic saline with 5% ethanol and 5% tween-20) was applied to activate TRPV1 cation channels selectively expressed on afferent fibers, or 3) sham denervation, in which the renal artery and vein were visualized (Foss et al., 2015). During bilateral renal denervation and selective afferent renal nerve ablation, care was taken to isolate the dissected neurovascular bundle from the surrounding tissue and excess solution was dried carefully to avoid off-target exposure to phenol and capsaicin, respectively. The flank incision was then sutured closed and the procedure was repeated on the contralateral side.

In Vivo Studies in Conscious Animals

Assessment of NCC activity

Rats underwent acute cannulation of the femoral vein, femoral artery, and bladder followed by a 2-hour surgical recovery period during which rats returned to full consciousness and stable cardiovascular and renal function. At the close of the 2-hour recovery, rats underwent an acute NCC activity assessment consisting of a 1-hour control period (isotonic saline, 20 μ L/min), a 1-hour ENaC blockade period (amiloride, ENaC antagonist; 2mg/kg bolus followed by 2mg/kg/hour at a rate of 20 μ L/min), and a 1-hour NCC blockade period during which ENaC blockade

was maintained (hydrochlorothiazide, NCC antagonist; 2mg/kg bolus followed by 2mg/kg/hour hydrochlorothiazide combined with 2mg/kg/hour amiloride at a total rate of 20 μ L/min) (Ashek et al., 2012; Walsh et al., 2016). Urine was collected in consecutive 10-minute periods for the assessment of renal sodium excretion. Baseline blood pressure and heart rate were assessed as the average during the 1-hour control period. Activity of the ENaC was assessed as the peak natriuretic response to amiloride, calculated by subtracting baseline natriuresis during the final two 10-minute periods of the control period from the maximal natriuresis during ENaC blockade. Similarly, NCC activity was assessed as the peak natriuretic response to hydrochlorothiazide, calculated by subtracting baseline natriuresis during the final two 10-minute periods of ENaC blockade from the maximal natriuresis during NCC blockade (during which ENaC blockade was maintained). For both amiloride and hydrochlorothiazide, maximal natriuresis occurred within the first two 10-minute periods of blockade.

Acute volume expansion

Rats underwent acute femoral vein, femoral artery, and bladder cannulation with or without renal artery cannulation followed by a 2-hour surgical recovery period during which rats returned to full consciousness and stable cardiovascular and renal function. At the close of the 2-hour recovery period, conscious rats underwent an acute intravenous volume expansion protocol consisting of a 20-minute baseline period (isotonic saline, 20 μ L/min) followed by a 30-minute volume

expansion (isotonic saline infused at a rate calculated to deliver 5% of the rat's body weight over 30 minutes), and a 90-minute recovery period (isotonic saline, 20 μ L/min) (Wainford & Kapusta, 2010; Kapusta et al., 2012). Mean arterial pressure and heart rate were monitored continuously and urine was collected in consecutive 10-minute increments throughout the protocol. At the close of the experimental protocol, all rats underwent transcatheter perfusion; in the subset of rats in which the renal artery was cannulated, the pressor response to intrarenal bradykinin was assessed to validate selective afferent renal nerve ablation prior to perfusion. Perfused brain and kidney tissue were collected for Fos immunohistochemistry and assessment of renal norepinephrine content and renal pelvic calcitonin gene-related peptide to further validate the efficacy and selectivity of selective afferent renal nerve ablation or bilateral renal denervation. In separate sets of rats, perfusion was performed immediately following the 2-hour surgical recovery and a 20-minute control period and perfused brains were collected for the assessment of baseline Fos expression.

Acute 1M NaCl infusion

Rats underwent acute femoral vein, femoral artery, and bladder cannulation with or without renal artery cannulation followed by a 2-hour surgical recovery period during which rats returned to full consciousness and stable cardiovascular and renal function. At the close of the 2-hour recovery period, conscious rats underwent an acute intravenous 1M NaCl infusion protocol consisting of a 1-hour

control period (isotonic saline, 20 μ L/min), followed by a 2-hour 1M NaCl infusion during which the rate of delivery was not altered (1M NaCl, 20 μ L/min) (Wainford et al., 2013). At the close of the experimental protocol, all rats underwent transcardiac perfusion; in the subset of rats that underwent renal artery cannulation, the pressor response to bradykinin was assessed to validate selective afferent renal nerve ablation prior to perfusion. Perfused brain and kidney tissue were collected for Fos immunohistochemistry and assessment of renal norepinephrine content and renal pelvic calcitonin gene-related peptide to further validate the efficacy and selectivity of selective afferent renal nerve ablation or bilateral renal denervation. In separate sets of rats, perfusion was performed immediately following the 2-hour surgical recovery and a 20-minute control period and perfused brains were collected for the assessment of baseline Fos expression.

*Assessment of glomerular filtration rate and renal blood flow
during acute sodium challenge*

In select groups of rats that underwent acute volume expansion or 1M NaCl infusion, glomerular filtration rate and renal blood flow were assessed beginning 30 minutes after the start of the surgical recovery period. Rats were infused with inulin (300mg/kg/hour) and para-amminohippurate (40mg/kg/hour) during the remaining 90 minutes of the 2-hour surgical recovery period and throughout the entire experimental protocol. In these animals, urine collection was performed as described above and blood was collected in the middle of each half-hour period.

Transcutaneous assessment of glomerular filtration rate

In select groups of rats that underwent acute volume expansion, 1M NaCl infusion, or assessment of NCC activity, glomerular filtration rate was assessed using a preclinical transdermal glomerular filtration rate monitor (Medibeacon GmbH, Mannheim, Germany) capable of exciting a fluorescent label and absorbing subsequent emissions (Schock-Kusch et al., 2011). Following acute cannulation of the femoral vein, femoral artery, and bladder, sodium methohexital anesthesia was briefly maintained while the monitor was secured on a depilated region on the back of the rat. An intravenous bolus of FITC-sinistrin (5mg per 100g body weight), a commercially available glomerular filtration rate marker modified with a fluorescent label, was administered 30 minutes after the start of the surgical recovery period, and the optical device was recovered at the end of the acute experimental protocol. The elimination kinetics of FITC-sinistrin were analyzed using proprietary software supplied with the transdermal monitor (Medibeacon GmbH, Mannheim, Germany) and glomerular filtration rate was calculated using the previously established formula below (Schock-Kusch et al., 2011):

$$GFR [ml/ min /100g b.w.] = \frac{31.26 [ml/100g b.w.]}{t_{1/2} (FITC - sinistrin)[min]}$$

In vivo validation of selective afferent renal nerve ablation

In select groups of rats that underwent selective afferent renal nerve ablation or sham denervation, the renal artery was cannulated following acute

cannulation of the femoral vein, femoral artery, and bladder prior to an acute volume expansion or 1M NaCl infusion. In these animals, the efficacy of selective afferent renal nerve ablation was confirmed in vivo as the absence of a pressor response to intrarenal bradykinin (Foss et al., 2015; Foss et al., 2016). Immediately following the completion of the volume expansion protocol or the 1M NaCl infusion protocol, and prior to transcardiac perfusion, mean arterial pressure and heart rate were recorded during a 5-minute baseline and a 5-minute intrarenal bradykinin infusion (40 μ g/kg/min). The pressor response to bradykinin was calculated as the difference in blood pressure between the baseline period and the final 2 minutes of the bradykinin infusion period.

In vivo validation of adrenoceptor blockade

In rats that underwent acute femoral vein, femoral artery, and bladder cannulation following a chronic subcutaneous infusion of an α_1 - or β -adrenoceptor antagonist alone, the cardiovascular responses to an intravenous bolus of phenylephrine (selective α_1 -adrenoceptor agonist; 4 μ g in 100 μ L) and a subsequent bolus of isoproterenol (non-selective β -adrenoceptor agonist; 0.7 μ g in 100 μ L) were assessed prior to sacrifice at the end of the acute in vivo protocol (Walsh et al., 2016). Selective α_1 -adrenoceptor blockade was confirmed as the absence of a pressor response to phenylephrine with an intact tachycardic response to isoproterenol, while selective β -adrenoceptor blockade was confirmed

as the absence of a tachycardic response to isoproterenol with an intact pressor response to phenylephrine.

Assessment of peak depressor response to ganglionic blockade

In subsets of animals that underwent acute femoral vein, femoral artery, and bladder cannulation, the peak depressor response to ganglionic blockade (hexamethonium, 30mg/kg intravenous bolus) was assessed as a measure of vascular sympathetic tone (Walsh et al., 2016). The peak depressor response was calculated as the difference between the lowest blood pressure achieved, which occurred within 5 minutes of the bolus, and baseline blood pressure, defined as the average blood pressure during the 10 minutes preceding bolus delivery.

Metabolic balance studies

Rats were housed individually in metabolic cages designed to separate fecal matter and food waste from urine for collection. Rats were allowed to acclimatize to the cages with ad libitum access to tap water and food for 48-hours prior to experimental data collection. During data collection periods, food and water consumption were measured in 24-hour periods and 24-hour urine was collected into beakers containing mineral oil to prevent evaporation over the course of each day. Daily sodium balance was calculated as the difference between dietary sodium intake and urinary sodium excretion (Kapusta et al., 2013; Wainford et al.,

2015; Walsh et al., 2016). At the end of the dietary manipulation protocol, rats were sacrificed and kidney tissue and plasma were collected for further analysis.

Radiotelemetry studies

Rats underwent radiotelemetry probe implantation followed by a 5-7 day surgical recovery period prior to the start of baseline experimental data collection. During experimental data collection, blood pressure data were recorded by radiotelemetry via scheduled sampling for 10 seconds every 10 minutes (Walsh et al., 2016). Data were collected, stored, and analyzed using Dataquest A.R.T. 4.2 software (DSI, MN). At the end of the dietary manipulation protocol, rats were sacrificed and kidney tissue and plasma were collected for further analysis.

In Vivo Studies in Anesthetized Animals

Manipulation of renal pelvic pressure

Rats that underwent renal pelvis cannulation were maintained under intravenous sodium thiopental anesthesia (0.04mmol/kg/hour at a rate of 50 μ L/min in isotonic saline) for a 90-minute rest period prior to manipulation of renal pelvic pressure to allow collection of stable steady-state urine. A fluid-filled syringe was connected to the renal pelvis cannula and elevated to increase renal pelvic pressure across a physiological range in 2.5mmHg increments across a physiological range of 0-10mmHg. Renal pelvic pressure was maintained for 10 minutes per increment and each incremental period was followed by a 10-minute

recovery period at 0mmHg. Renal pelvic pressure was recorded continuously and contralateral urine was collected during each 10-minute incremental period.

Manipulation of renal pelvic sodium concentration

Rats that underwent renal pelvis cannulation were maintained under intravenous sodium thiopental anesthesia (0.04 mmol/kg/hour at a rate of 50 μ L/min in isotonic saline) for a 90-minute rest period prior to manipulation of renal pelvic pressure to allow collection of stable steady-state urine. An infusion pump was connected to the renal pelvis cannula and used to deliver a 10-minute infusion of isotonic (154mM) saline at a rate of 20 μ L/min followed by a 10-minute infusion of hypertonic (450mM) saline at the same rate, such that renal pelvic pressure was not altered throughout the course of the experiment. Renal pelvic pressure was recorded continuously and contralateral urine was collected during each 10-minute incremental period.

Ex Vivo Studies

Ex vivo renal pelvis assay

Rats underwent conscious decapitation and the renal pelvis from each kidney was carefully dissected. Each renal pelvis was placed individually into a separate well of a 24-well plate containing 400 μ L HEPES and maintained at 37°C. The medium was aspirated and replaced every 10 minutes during a 2-hour equilibration period prior to experimentation. The responsiveness of the afferent

renal nerves was then assessed as the release of substance P in response to norepinephrine, a general stimulus that acts at α_1 -adrenoceptors to activate the afferent renal nerves, or hypertonic saline, a chemoreceptor stimulus (Kopp et al., 2007; Kopp et al., 2009; Kopp et al., 2011a; Kopp et al., 2011b). In brief, the medium was collected for storage and replaced every 5 minutes during four control periods (HEPES), one experimental treatment period (1250pM norepinephrine or 450mM NaCl in HEPES), and four recovery periods (HEPES). As two renal pelvises were available from each rat, each rat served as its own internal control with one renal pelvis treated with 1250pM norepinephrine and the other treated with 450mM NaCl. The medium collected during control, experimental treatment, and recovery periods was stored at -80°C prior to analysis of substance P content via ELISA (ADI-901-018, Enzo Life Sciences, NY). Norepinephrine- and sodium-evoked substance P release were calculated as the difference in substance P release between the experimental treatment period and the average of the four control periods.

Molecular and Analytical Techniques

Urinalysis

Urine volume was determined gravimetrically assuming 1g = 1mL. Urinary sodium concentration was assessed using flame photometry (IL-943, Instrumentation Laboratories, MA).

Biochemical validation of selective afferent renal nerve ablation

The efficacy and selectivity of selective afferent renal nerve ablation versus bilateral renal denervation were confirmed in kidney tissue via the assessment of renal pelvic calcitonin gene-related peptide content and renal norepinephrine content (calcitonin gene-related peptide ELISA #589001, Cayman Chemicals, MI; NE ELISA IB89537, IBL America, MN). Selective afferent renal nerve ablation is reflected by a loss of renal pelvic calcitonin gene-related peptide but not renal norepinephrine content (Foss et al., 2015).

Biochemical validation of bilateral renal denervation

The efficacy of bilateral renal denervation was confirmed via the assessment of renal norepinephrine content (NE ELISA IB89537, IBL America, MN). Bilateral renal nerve ablation is reflected by a reduction in renal norepinephrine content.

Fos immunohistochemistry following acute sodium challenge

In rats that underwent acute volume expansion or 1M NaCl infusion, or were sacrificed immediately following completion of the 2-hour recovery and control periods, Fos expression was assessed in the paraventricular nucleus as previously described (Randolph et al., 1998; Carmichael et al., 2016). Upon completion of the in vivo study, rats were anesthetized using sodium methohexital (10mg/kg administered intravenously) and underwent transcardiac perfusion with 0.1M

phosphate buffered saline (150-200mL) followed by 4% paraformaldehyde in 0.1M phosphate buffered saline (200-300mL). Brains were dissected and fixed overnight in 4% paraformaldehyde at 4°C followed by cryo-preservation in a 30% weight/volume sucrose solution for 48 hours, replacing the sucrose solution once after 24 hours. The brain was then embedded in OCT Tissue Tek, frozen using dry ice, and sectioned coronally on a cryostat using a rat brain atlas to identify the rostral and caudal limits of the paraventricular nucleus. The paraventricular nucleus was serially sectioned into three sets of 40µm sections, each set spanning the entire paraventricular nucleus. Sections were stored at -20°C in cryoprotectant (30% weight/volume sucrose, 30% ethylene glycol, and 1% polyvinyl-pyrrolidone in 0.1M PBS) (Watson et al., 1986).

Fos immunohistochemistry was then performed on one set of free-floating sections from each brain (Carmichael et al., 2016). Sections stored in cryoprotectant were allowed to come to room temperature and immunohistochemistry was performed using an anti-Fos primary antibody (Ab-5, Calbiochem, CA) and a biotinylated horse anti-rabbit IgG secondary antibody (Vector Laboratories, CA) according to the steps described in Table 2.3. Sections incubated in antibody diluent without the primary antibody were also processed and imaged to ensure that Fos staining reflected primary antibody binding.

Step/Purpose	Solution	Conditions
Wash	0.1M PBS	4 x 5 minutes, RT
Block endogenous peroxidases	0.3% hydrogen peroxide	30 minutes, RT
Wash	0.1M PBS	4 x 5 minutes, RT
Block non-specific binding	3% normal horse serum 0.25% Triton X-100	2 hours, RT
Primary antibody	Anti-Fos 1:30,000 in blocking solution	1 hour, RT prior to 48 hours, 4°C
Wash	0.1M PBS	4 x 5 minutes, RT
Biotinylated secondary antibody	Biotinylated horse anti-rabbit IgG 1:200 in blocking solution	2 hours, RT
Wash	0.1M PBS	4 x 5 minutes, RT
Peroxidase labeling/ amplification	Avidin-peroxidase conjugate (ABC- Vectastain Kit; Vector Laboratories)	1 hour, RT
Wash	0.1M PBS	4 x 5 minutes, RT
Chromogenic detection	0.04% 3,3'-diaminobenzidine hydrochloride 0.04% nickel ammonium sulphate	11 minutes, RT
Wash	0.1M PBS	4 x 5 minutes, RT

Table 2.3. Detailed protocol for Fos immunohistochemistry. All solutions are in 0.1M phosphate buffered saline (PBS) unless otherwise noted. RT = room temperature.

Stained sections were then mounted onto gelatin-coated slides and dehydrated via a graded series of alcohols followed by xylenes. Slides were coverslipped using Permount mounting medium and imaged using an Olympus microscope (BX41) and an Olympus DP70 digital camera with DP MANAGER software (v 2.2.1) (Olympus, PA, USA). For each animal, Fos-positive cells were counted in two sections from each of 3 rostral-caudal levels using ImageJ (NIH, MD) and the counts for each subnucleus of the paraventricular nucleus were averaged.

Assessment of renal cortex protein expression

Kidneys were harvested and the renal cortex and pelvis were carefully dissected and stored separately at -80°C prior to isolation of a renal cortical membrane preparation and assessment of protein expression as previously described (Walsh et al., 2016). Approximately 200mg of renal cortex from each animal was homogenized on ice via hand-pestle in ice-cold homogenizing buffer with protease inhibitors (10mM triethanolamine, 250mM sucrose, 100mM NaN₃, 10mM PMSF, and 1mM leupeptin) and then centrifuged at 4,000g for 10 minutes at 4°C. The pellet containing unhomogenized tissue and nuclei and mitochondria from homogenized tissue was discarded, while the supernatant was isolated and centrifuged again at 17,000g for 1 hour at 4°C to yield a pellet containing the plasma membrane fraction. The pellet was resuspended in cell lysis buffer (9803S, Cell Signaling Technology, MA) and a bicinchoninic acid protein assay (BCA Protein Assay, Thermo Scientific Pierce, IL) was performed to quantify total protein content. The membrane preparation was stored at -80°C until use in immunoblotting. Immunoblotting was conducted using 20µg of protein per lane in 7.5% or 10% SDS-PAGE gels. Proteins were transferred from the gels to nitrocellulose membranes that were then blocked in 5% milk for 30 minutes prior to overnight incubation at 4°C in primary antibody (Table 2.4). Membranes were then washed and incubated in secondary antibody (Table 2.4) for 1 hour at room temperature prior to visualization using chemiluminescence.

Antibody	Company	Item number	Dilution
Anti-NCC	Millipore	AB3553	1:2000
Anti-pNCC (pT53)	Phosphosolutions	P1311-53	1:1000
Anti-pNCC (pT58)	Obtained from Robert Fenton	None	1:750
Anti-WNK1	Santa Cruz	Sc-28897	1:200
Anti-OxSR1	Abcam	Ab125468	1:1000
Anti-SPAK	Abcam	Ab79045	1:1000
Anti-pOxSR1 (pT185)	Abcam	Ab138655	1:1000
Anti-pSPAK (Ser373) / pOxSR1 (Ser325)	Millipore	07-2273	1:1000
Anti- β -actin	Sigma	A5316	1:5000
Donkey Anti-Rabbit IgG H&L (HRP)	Abcam	Ab16284	1:2500
Anti-Mouse IgG Peroxidase	Sigma	A9044	1:10000

Table 2.4. Antibody dilutions used for immunoblotting. NCC = sodium chloride cotransporter; pNCC (pT53) = phosphorylated NCC (phospho-Thr53); WNK1 = with no lysine kinase 1; OxSR1 = oxidative stress responsive kinase 1; SPAK = STE20/SPS1-related Proline-Alanine-rich kinase; HRP = horseradish peroxidase

Membranes were imaged using an ImageQuant LAS 4000 imager (GE Healthcare Life Sciences, MA) and semi-quantified using Image J (NIH, MD). Protein levels were normalized to β -actin abundance assessed using the same membrane or total protein loading assessed using coomassie blue staining of a gel run simultaneously with the same samples.

Assessment of plasma norepinephrine concentration

Plasma norepinephrine was assessed via ELISA (IB89552, IBL America, MN) according to manufacturer's directions.

Assessment of renal norepinephrine content

Renal norepinephrine content was assessed via ELISA (IB89537, IBL America, MN) according to manufacturer's directions.

Assessment of urinary kidney injury molecule-1 concentration

Urinary kidney injury molecule-1 was assessed via ELISA (ab119757, Abcam, MA) according to manufacturer's directions.

Assessment of proteinuria

Urinary protein content was assessed via bicinchoninic acid protein assay (BCA Protein Assay, Thermo Scientific Pierce, IL) according to manufacturer's directions.

Assessment of urinary angiotensinogen

Urinary angiotensinogen was assessed via ELISA (IB27414, IBL America, MN) according to manufacturer's directions.

Assessment of plasma renin activity

Plasma renin activity was assessed as generated angiotensin I via ELISA (IB59131, IBL America, MN) according to manufacturer's directions.

Assessment of plasma angiotensin II

Plasma angiotensin II content was assessed via ELISA (ADI-900-204, Enzo Life Sciences, NY) according to manufacturer's directions.

Assessment of plasma aldosterone

Plasma aldosterone content was assessed via ELISA (ADI 901-173, Enzo Life Sciences, NY) according to manufacturer's directions.

Assessment of glomerulosclerosis and mesangial expansion

Kidneys were bisected and immersed in neutral buffered formalin. The tissue was stored in formalin at room temperature until processing, embedding, and Periodic Acid-Schiff staining with a hematoxylin counterstain were performed by the Boston University School of Medicine Experimental Pathology Laboratory Service Core. Approximately 40 glomeruli per animal were imaged at 60X magnification using an Olympus microscope (BX41) and an Olympus DP70 digital camera with DP MANAGER software (v 2.2.1) (Olympus, PA, USA). Glomerulosclerosis and mesangial expansion, two pathological changes observed in human aging (Zhou et al., 2008), were evaluated and scored separately by two blinded reviewers (Raij et al., 1984; Maric et al., 2004). The scores noted by each reviewer were averaged. Glomerulosclerosis was defined as the obliteration of capillary lumens, folding and thickening of the glomerular basement membrane, and loss of cellular elements from the glomerular tuft, while mesangial expansion

was defined as the present of periodic acid-Schiff-positive material in the mesangium. Semi-quantitative scores were obtained using a previously established scale in which the percentage of each individual glomerulus involved was denoted as 0 (no lesion) or 1, 2, 3, or 4, corresponding to 25%, 50%, 75%, and 100% glomerular area involvement. An overall score for each measure was then calculated for each animal using the formula below:

$$\text{OVERALL SCORE} = \frac{[(\# \text{ glomeruli scored "0"}) \times 0] + [(\# \text{ glomeruli scored "1"}) \times 1] + [(\# \text{ glomeruli scored "2"}) \times 2] + [(\# \text{ glomeruli scored "3"}) \times 3] + [(\# \text{ glomeruli scored "4"}) \times 4]}{\text{Total number of glomeruli scored}} \times 100$$

Assessment of substance P release in the ex vivo renal pelvic assay

Substance P concentration in supernatant generated during the ex vivo renal pelvis assay was assessed via ELISA (ADI-901-018, Enzo Life Sciences, NY) according to manufacturer's directions. Norepinephrine- and sodium-evoked substance P release were calculated as the difference in substance P release between the experimental treatment period and the average of the four control periods.

Statistical Analysis

All data in Chapters 3-5 are expressed as mean \pm SEM. The magnitude of change in cardiovascular and renal excretory parameters at different time points after initiation of acute sodium challenge or chronic dietary sodium intake was compared with the average group control value by a one-way repeated-measures

analysis of variance (ANOVA) with subsequent Dunnett's test. Differences between treatment groups or age groups were assessed by a two-way repeated measure ANOVA, with treatment or age being one fixed effect and time the other, with the interaction included. The time (minutes) was used as the repeated factor. Post-hoc analysis was performed using Bonferroni's test to compare variations among the groups. Statistical analysis was carried out using Prism 7 (GraphPad Software, CA). In all studies, statistical significance was defined as $P < 0.05$.

CHAPTER THREE: Adrenergic Regulation of the NCC in Salt-Sensitive Hypertension

Abstract

Salt sensitivity of blood pressure is characterized by inappropriate sympathoexcitation and renal sodium reabsorption during high salt intake, and exogenous norepinephrine infusion promotes salt-sensitive hypertension and prevents dietary sodium-evoked suppression of sodium chloride cotransporter (NCC) activity in classically salt-resistant animal models. Studies of the adrenergic signaling pathways that promote NCC activity during norepinephrine infusion have yielded conflicting results implicating α_1 - and/or β -adrenoceptors and a downstream kinase network that phosphorylates and activates the NCC, including with no lysine kinases (WNKs), STE20/SPS1-related Proline-Alanine-rich kinase (SPAK), and oxidative stress responsive kinase 1 (OxSR1). In these studies, we used selective adrenoceptor antagonism in norepinephrine-infused male Sprague Dawley rats to investigate the differential roles of α_1 - and β -adrenoceptors in NCC regulation. Norepinephrine infusion evoked salt-sensitive hypertension and prevented dietary sodium-evoked suppression of NCC mRNA and protein expression, phosphorylation, and in vivo activity. Impaired NCC suppression during high salt intake in norepinephrine-infused rats was paralleled by impaired suppression of WNK1, SPAK, and OxSR1 expression and an increase in OxSR1 phosphorylation. Antagonism of α_1 -adrenoceptors initiated prior to high salt intake or after the establishment of salt-sensitive hypertension restored dietary sodium-

evoked suppression of the NCC and its regulatory kinases and abolished the salt-sensitive component of hypertension. In contrast, β -adrenoceptor antagonism attenuated norepinephrine-evoked hypertension independently of dietary sodium intake and did not restore the suppression of the NCC or its regulatory kinases during high salt intake. Together, these findings suggest that a selective α_1 -adrenoceptor-gated WNK1/OxSR1 signaling pathway prevents dietary sodium-evoked NCC suppression, promoting the development and maintenance of salt-sensitive hypertension.

Introduction

Dietary sodium intake plays a significant role in blood pressure regulation, as increased sodium retention is a well-established precursor of hypertension (Kannel, 2000; World Health, 2013; Olsen et al., 2016). Approximately 50% of hypertensive individuals and 25% of normotensive individuals exhibit the salt sensitivity of blood pressure (Morimoto et al., 1997; Appel et al., 2011), a phenomenon that can promote the development of salt-sensitive hypertension. Multiple factors contribute to the development of salt-sensitive hypertension including increased renal sodium reabsorption. Given that salt sensitivity is driven, in part, by an inappropriate sympathoexcitatory response to dietary sodium intake (Brooks et al., 2005; Stocker et al., 2013) that drives an increase in salt and water retention, there is a pivotal role of the kidney in the pathophysiology of salt-sensitive hypertension.

The sodium chloride cotransporter (NCC), a thiazide-sensitive transporter predominantly found on the apical side of the distal convoluted tubule, contributes to the fine-tuning of sodium reabsorption (Huang & Cheng, 2015; Hadchouel et al., 2016; Shekarabi et al., 2017). Multiple recent studies have suggested a direct influence of the sympathetic nervous system release of norepinephrine on the expression and activity of the NCC. In salt-resistant Sprague-Dawley rats, increased dietary salt intake suppresses sympathetic outflow and circulating norepinephrine levels (Kapusta et al., 2012; Kapusta et al., 2013; Wainford et al., 2015) and persistently reduces the expression and activity of the NCC (Sandberg et al., 2006; Walsh et al., 2016). Subcutaneous norepinephrine infusion during normal salt intake appears to evoke a species-specific NCC response with no alteration in NCC expression in Sprague-Dawley rats (Sonalker et al., 2008; Walsh et al., 2016) versus an increase in total and phosphorylated NCC in C57Bl/6J mice (Mu et al., 2011; Uchida et al., 2012).

The regulation of the NCC involves a complex kinase network including with no lysine kinases WNK1 and WNK4, STE20/SPS1-related Proline-Alanine-rich kinase (SPAK), and oxidative stress responsive kinase 1 (OxSR1) (Huang & Cheng, 2015; Hadchouel et al., 2016; Shekarabi et al., 2017). The regulation of the NCC by norepinephrine remains an area of controversy. It was initially reported that norepinephrine acts via a β 2-adrenoceptor-gated WNK4-mediated signal transduction pathway to evoke long-term upregulation of the NCC, driving sodium reabsorption and salt-sensitive hypertension in mice (Mu et al., 2011).

Significantly, the proposed β 2-WNK4-NCC signaling pathway in salt-sensitive hypertension was questioned following the failure to reproduce the role of WNK4 in the development of salt-sensitive hypertension in norepinephrine-infused mice (Uchida et al., 2012). Further, a recent study demonstrated that following direct pharmacological stimulation, α ₁- and β - adrenoceptors act synergistically to drive acute increases in NCC expression (Terker et al., 2014). At present the mechanisms by which long-term alterations in norepinephrine levels, such as those seen in animal models of salt-resistance or salt-sensitivity, influence NCC regulation remain to be fully delineated.

Our prior study, conducted in salt-resistant Sprague Dawley rats, did not investigate the mechanisms or signal transduction pathways by which norepinephrine influences chronic NCC activity during long-term alterations in dietary salt intake. As such, we hypothesized that in salt-sensitive hypertension, sympathoexcitation increases NCC-mediated activity via an adrenoceptor-mediated signal transduction pathway that increases the expression and/or activity of NCC regulatory kinases. To test our hypothesis, we used norepinephrine-infused male Sprague Dawley rats as a model of sympathetically-mediated salt-sensitive hypertension (Walsh et al., 2016). We administered a chronic norepinephrine infusion during normal and high dietary salt intake and used co-infusions of terazosin (α ₁-adrenoceptor antagonist) or propranolol (β -adrenoceptor antagonist) to provide novel mechanistic insight into the adrenergic signaling

pathways regulating NCC activity and expression during both the development and maintenance of salt-sensitive hypertension.

Methods

All methods used in the studies of adrenergic regulation of the NCC in young adult animals in this chapter are briefly outlined in this section and described in further detail in Chapter 2. In brief, 3 month old male Sprague Dawley rats were used as an established model of salt resistance in which norepinephrine infusion evokes the development of salt-sensitive hypertension.

To validate previous physiological findings, miniosmotic pumps delivering isotonic saline or norepinephrine infusions were placed in animals immediately prior to the start of a 21-day normal or high salt diet and blood pressure and in vivo NCC activity were assessed on day 21 in conscious animals (Figure 3.1A). Extending previous studies with mechanistic insight into the signaling pathways mediating NCC regulation in norepinephrine-infused rats, mRNA and protein expression and phosphorylation of the NCC and its regulatory kinases WNK1, SPAK, and OxSR1 were assessed in renal cortex tissue isolated from these animals immediately following the acute study (Figure 3.1A).

To determine the role of α_1 - and β -adrenoceptor signaling pathways in norepinephrine-evoked salt-sensitive hypertension and NCC regulation, the same studies were performed in animals receiving infusions of terazosin (α_1 -adrenoceptor antagonist) or propranolol (β -adrenoceptor antagonist) alone or in

combination with norepinephrine during a 21-day normal or high salt diet (Figure 3.1A).

To determine whether α_1 -adrenoceptor antagonism is capable of attenuating established salt-sensitive hypertension and reversing established impairments in NCC regulation, miniosmotic pumps delivering isotonic saline or norepinephrine infusions were placed in animals immediately prior to the start of a 42-day high salt diet (Figure 3.1B). On day 21 of high salt intake, a timepoint at which norepinephrine-infused rats exhibit established salt-sensitive hypertension, the miniosmotic pump was replaced as follows: saline-infused rats received a new saline-filled pump, while sets of norepinephrine-infused rats received pumps delivering norepinephrine alone or in combination with terazosin (Figure 3.1B). The high salt diet was then continued for 21 days and blood pressure and NCC activity were assessed on cumulative day 42 of high salt intake (Figure 3.1B).

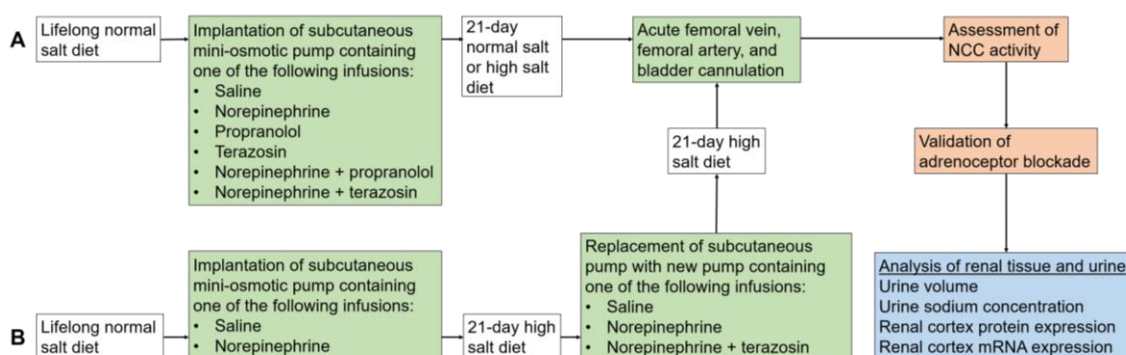


Figure 3.1. Overview of methods used in Chapter 3. All studies were performed using 3-month old male Sprague Dawley rats. White = dietary sodium assignments; green = surgical procedures; orange = in vivo studies in conscious animals; blue = analytical techniques. $n = 6/\text{group}$ unless otherwise specified. Detailed protocols are described in Chapter 2: General Methods.

Results

Chronic norepinephrine infusion evokes the salt sensitivity of blood pressure and prevents dietary sodium-evoked suppression of NCC activity, expression, and phosphorylation

In saline-infused rats, high salt intake had no impact on blood pressure and promoted the suppression of NCC activity, assessed as the natriuretic response to the NCC antagonist hydrochlorothiazide (Figure 3.2A&B). Dietary sodium-evoked suppression of NCC activity in vivo in saline-infused rats was paralleled by a reduction in NCC mRNA, protein expression, and phosphorylation at Thr58 (Figure 3.2C-E, Figure 3.3A).

Norepinephrine-infused rats maintained on a normal salt diet exhibited an increase in blood pressure that occurred independently of altered NCC activity (Figure 3.2A&B). A 21-day high salt diet exacerbated hypertension in norepinephrine-infused rats, indicating the development of salt-sensitive hypertension (Figure 3.2A&B). Critically, norepinephrine-evoked salt-sensitive hypertension was associated with a failure to suppress NCC activity during high salt intake (Figure 3.2A&B). Mirroring these findings, while norepinephrine had no impact on the NCC during normal salt intake, norepinephrine-infused rats failed to suppress NCC mRNA and total protein expression and NCC phosphorylation at Thr58 during high salt intake (Figure 3.2C-E, Figure 3.3A).

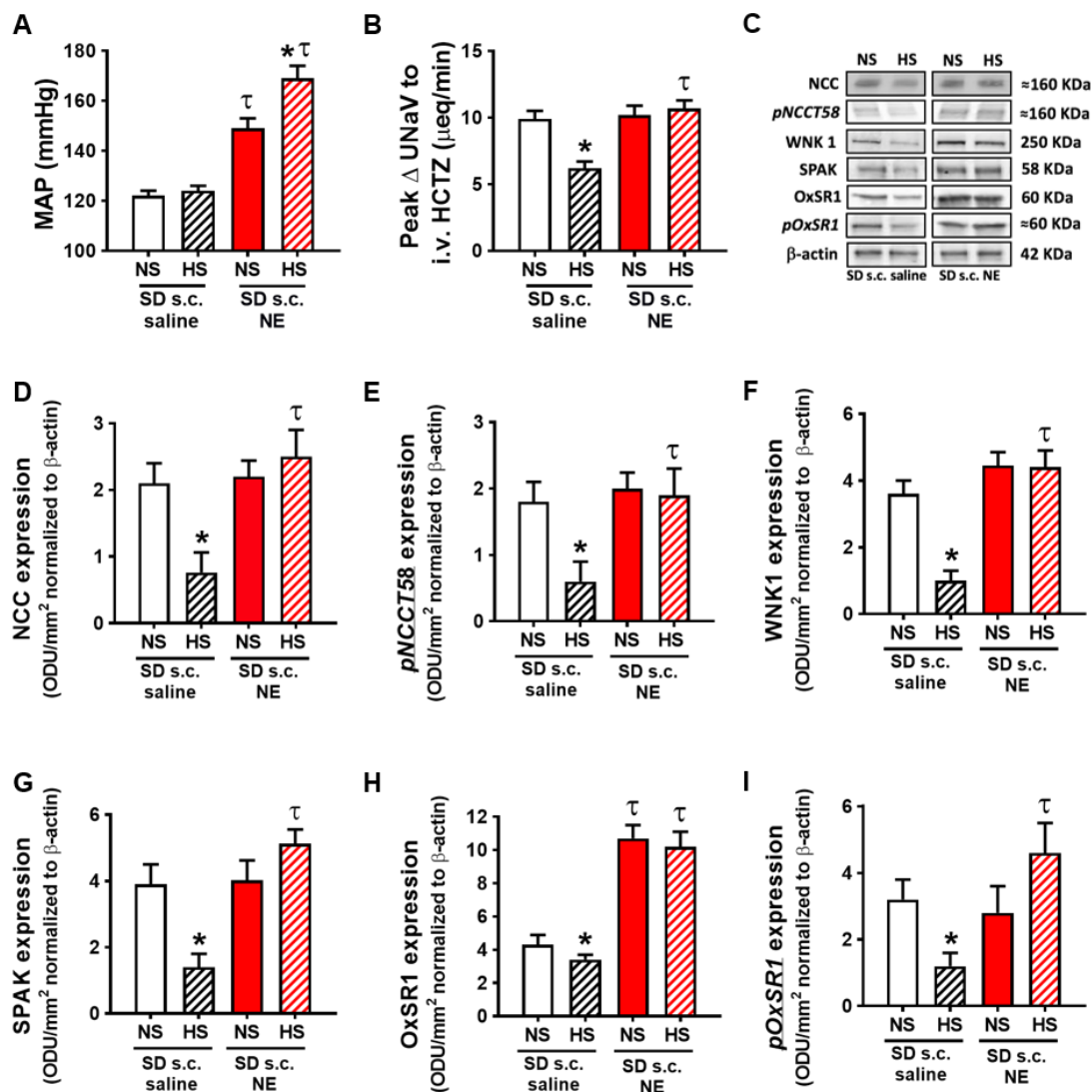


Figure 3.2. Impact of chronic norepinephrine infusion on blood pressure and NCC regulation. (A) Mean arterial pressure (MAP; mmHg) and (B) peak natriuretic response (Δ UNaV) to intravenous hydrochlorothiazide (HCTZ; 2mg/kg bolus, 2mg/kg hour infusion) in conscious 3-month old male Sprague Dawley (SD) rats receiving a subcutaneous (s.c.) infusion of saline or norepinephrine (NE) during a 21-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet and (C) representative immunoblots of renal cortical (D) total NCC expression, (E) NCC phosphorylation (Thr58), (F) WNK1 expression, (G) SPAK expression, (H) OxSR1 expression, and (I) OxSR1 phosphorylation. $n = 6$ /group. * $P < 0.05$ vs. respective NS group, $\tau P < 0.05$ vs. respective saline-infused group.

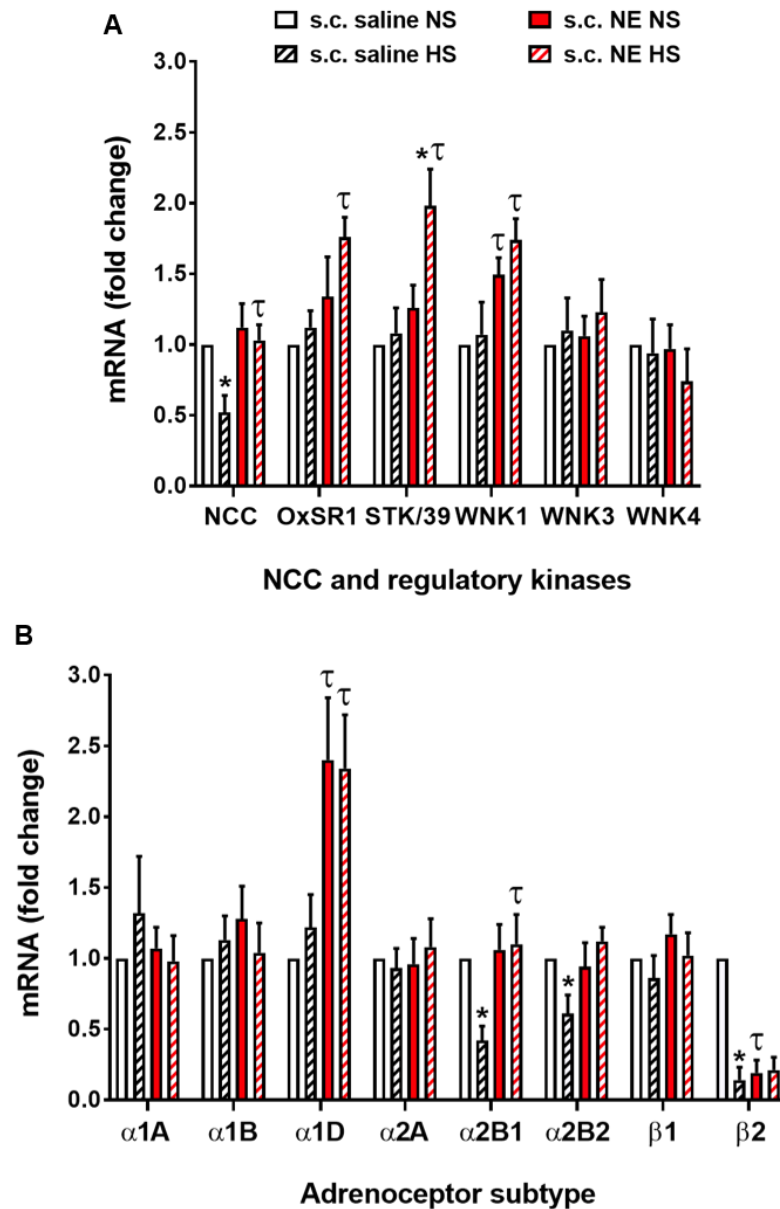


Figure 3.3. Impact of chronic norepinephrine infusion on mRNA expression of the NCC, its regulatory kinases, and adrenoceptor subtypes. Renal cortex mRNA expression of (A) the NCC and its regulatory kinases and (B) adrenoceptor subtypes in 3-month old male Sprague Dawley rats that received a subcutaneous (s.c.) infusion of saline or norepinephrine (NE) during a 21-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet. $n = 6/\text{group}$. * $P < 0.05$ vs. respective NS group, $\tau P < 0.05$ vs. respective saline-infused group.

Chronic norepinephrine infusion impairs the dietary sodium-evoked suppression of NCC regulatory kinases WNK1, SPAK, and OxSR1

Total protein expression of WNK1, SPAK, and OxSR1, as well as OxSR1 phosphorylation, were suppressed during high salt intake in saline-infused rats (Figure 3.2F-I). In contrast, while norepinephrine did not alter the expression of the NCC or its regulatory kinases during normal salt intake, impaired dietary sodium-evoked NCC suppression in norepinephrine-infused rats was accompanied by a failure to suppress WNK1, SPAK, and OxSR1 protein expression during high salt intake and a dietary sodium-evoked increase in OxSR1 phosphorylation (Figure 3.2F-I).

At the mRNA level, WNK1 expression was increased by norepinephrine infusion in rats maintained on a normal salt diet and remained elevated during high salt intake (Figure 3.3A). In contrast, WNK3 and WNK4 mRNA expression were not influenced by treatment or diet (Figure 3.3A). In norepinephrine-infused rats maintained on a 21-day high salt diet, OxSR1 mRNA expression was elevated compared to saline-infused rats during high salt intake, and SPAK mRNA expression was increased compared to both saline-infused rats during high salt intake and norepinephrine-infused rats during normal salt intake (Figure 3.3A). Interestingly, while mRNA expression of the α_{1A} , α_{1B} , α_{2A} , and β_1 -adrenoceptor subtypes was not altered by norepinephrine or high salt intake, saline-infused rats exhibited a dietary sodium-evoked suppression of α_{2B1} and α_{2B2} -adrenoceptors that was abolished by norepinephrine infusion (Figure 3.3B). Further,

norepinephrine infusion evoked an increase in α_{1D} -adrenoceptor mRNA expression to a similar degree during both normal and high salt intake, and norepinephrine and high salt intake both alone or in combination promoted a decrease in β_2 -adrenoceptor mRNA expression compared to saline-infused rats on a normal salt diet (Figure 3.3B).

Chronic α_1 -adrenoceptor antagonism prevents the development of norepinephrine-evoked salt sensitivity of blood pressure and restores dietary sodium-evoked NCC suppression

Chronic antagonism of α_1 -adrenoceptors using subcutaneous terazosin infusion alone had no impact on blood pressure or in vivo NCC activity in rats maintained on a normal salt diet (Figure 3.4A&B). Moreover, terazosin-infused rats maintained baseline blood pressure and retained the ability to suppress NCC activity during a 21-day high salt diet initiated immediately following the start of α_1 -adrenoceptor antagonism (Figure 3.4A&B). Significantly, while α_1 -adrenoceptor blockade did not alter blood pressure or NCC activity in norepinephrine-infused rats during normal salt intake, terazosin prevented the development of the salt-sensitive component of hypertension in norepinephrine-infused rats and restored dietary sodium-evoked NCC suppression in vivo (Figure 3.4A&B). In all rats, selective α_1 -adrenoceptor antagonism was confirmed as the loss of a pressor response to phenylephrine while the tachycardic response to isoproterenol remained intact (Figure 3.4C&D).

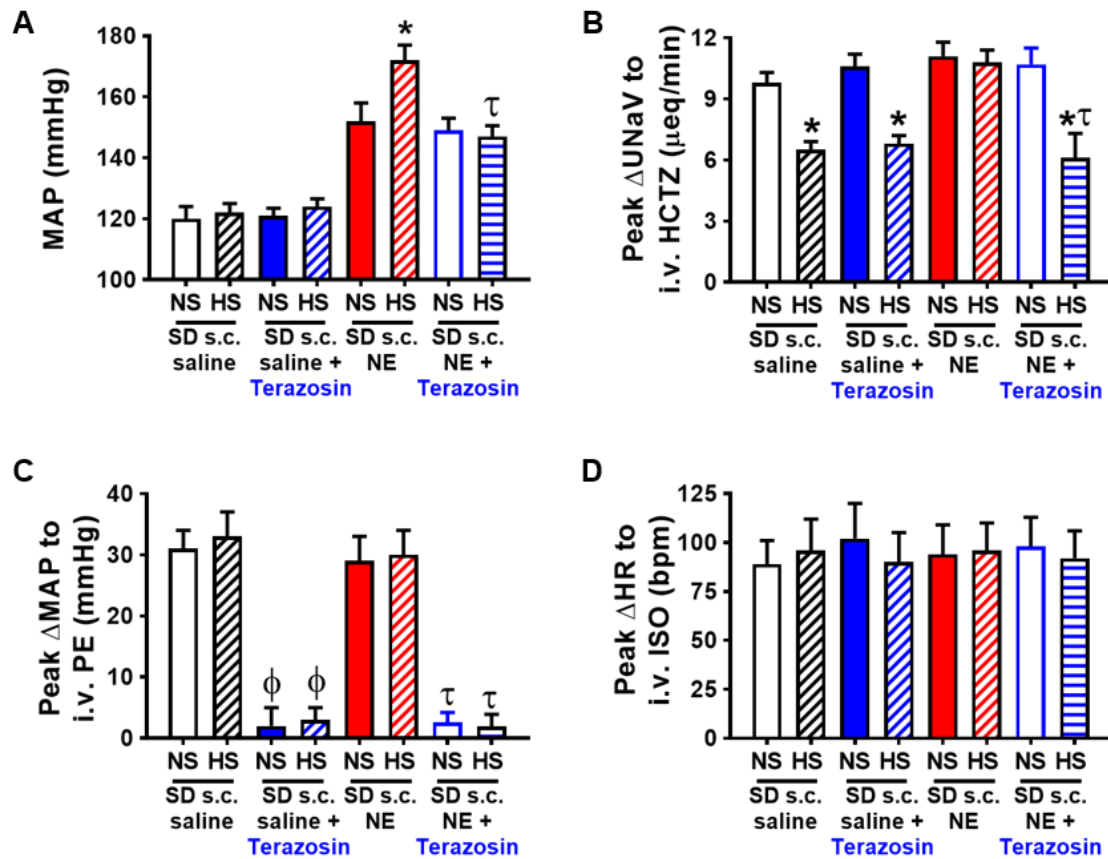


Figure 3.4. Impact of chronic α_1 -adrenoceptor antagonism on the development of norepinephrine-evoked salt-sensitive hypertension. (A) Mean arterial pressure (MAP; mmHg), (B) peak natriuretic response (Δ UNaV; μ eq/min) to intravenous (i.v.) hydrochlorothiazide (HCTZ; 2mg/kg bolus, 2mg/kg hour infusion), (C) peak pressor response (Δ MAP; mmHg) to i.v. phenylephrine (PE; 4 μ g/kg bolus), and (D) peak tachycardic response (Δ HR; beats per minute [bpm]) to i.v. isoproterenol (ISO; 0.7 μ g/kg bolus) in conscious 3-month old male Sprague Dawley (SD) rats that received a subcutaneous (s.c.) infusion of saline, saline + terazosin, norepinephrine (NE), or NE + terazosin during a 21-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet. $n = 6$ /group. * $P < 0.05$ vs. respective NS group, $\tau P < 0.05$ vs. respective NE-infused group, $\Phi P < 0.05$ vs. respective saline-infused group.

Chronic β -adrenoceptor antagonism attenuates hypertension but fails to restore dietary sodium-evoked NCC suppression

Chronic antagonism of β -adrenoceptors using subcutaneous propranolol infusion alone had no impact on blood pressure or NCC activity during normal or high salt intake (Figure 3.5A&B). In norepinephrine-infused rats, propranolol infusion reduced blood pressure to a similar level regardless of dietary sodium intake and had no impact on NCC activity during either diet compared to rats infused with norepinephrine alone, suggesting that norepinephrine-mediated NCC regulation occurs independently of β -adrenoceptor signaling (Figure 3.5A&B). In all rats, selective β -adrenoceptor antagonism was confirmed as the loss of a tachycardic response to isoproterenol while the pressor response to phenylephrine remained intact (Figure 3.5C&D).

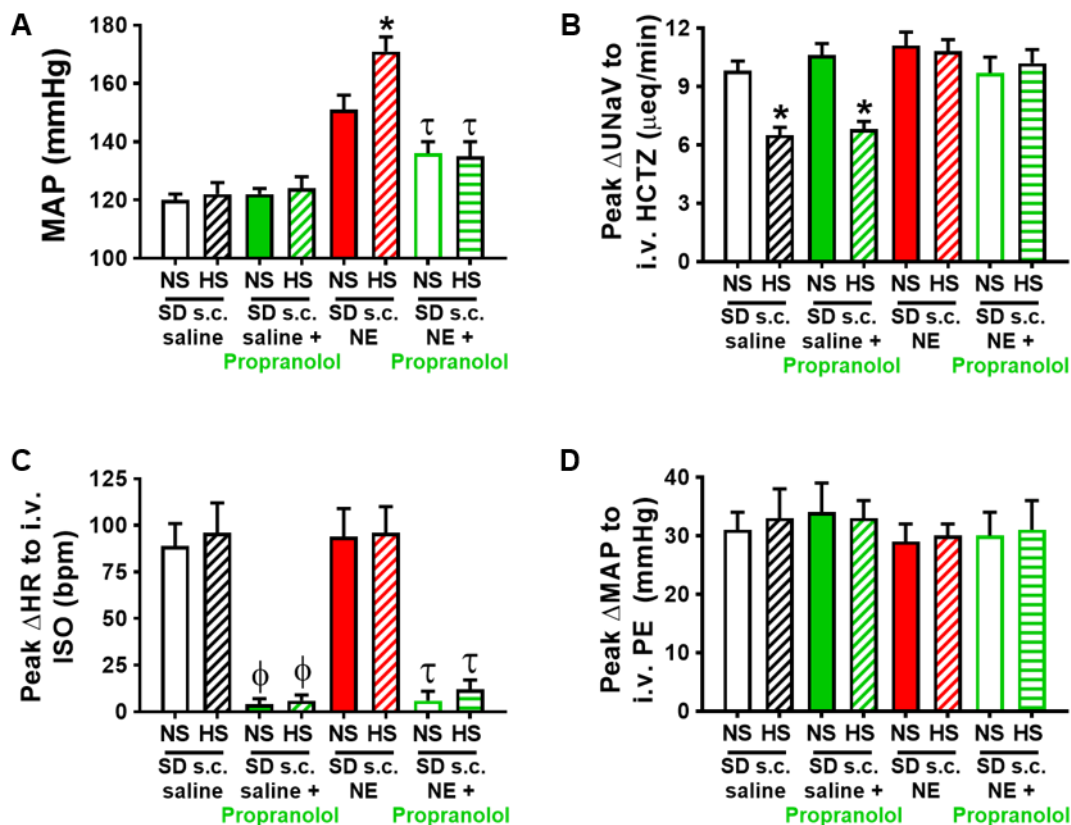


Figure 3.5. Impact of chronic β -adrenoceptor antagonism on the development of norepinephrine-evoked salt-sensitive hypertension. (A) Mean arterial pressure (MAP; mmHg), (B) peak natriuretic response (Δ UNaV; μ eq/min) to intravenous (i.v.) hydrochlorothiazide (HCTZ; 2mg/kg bolus, 2mg/kg hour infusion), (C) peak tachycardic response (Δ HR; beats per minute [bpm]) to i.v. isoproterenol (ISO; 0.7 μ g/kg bolus), and (D) peak pressor response (Δ MAP; mmHg) to i.v. phenylephrine (PE; 4 μ g/kg bolus) in conscious 3-month old male Sprague Dawley (SD) rats that received a subcutaneous (s.c.) infusion of saline, saline + terazosin, norepinephrine (NE), or NE + terazosin during a 21-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet. $n = 6$ /group. * $P < 0.05$ vs. respective NS group, $\tau P < 0.05$ vs. respective NE-infused group, $\phi P < 0.05$ vs. respective saline-infused group.

Chronic α_1 - and β -adrenoceptor antagonism differentially modulate the expression and phosphorylation of the NCC and its regulatory kinases

Terazosin infusion, which did not alter NCC activity during normal salt intake, prevented the norepinephrine-evoked increase in OxSR1 protein expression but otherwise had no impact on the NCC and its regulatory kinases at the protein level during normal salt intake (Figure 3.6A-F). Critically, the restoration of dietary sodium-evoked suppression of NCC activity by α_1 -adrenoceptor blockade was paralleled by restored suppression of NCC expression and phosphorylation during high salt intake (Figure 3.6A-B). Similarly, α_1 -adrenoceptor blockade restored dietary sodium-evoked suppression of WNK1 and SPAK protein expression and OxSR1 phosphorylation in norepinephrine-infused rats (Figure 3.6C-F). While these animals did not suppress OxSR1 protein expression during high salt intake compared to normal salt intake, OxSR1 protein expression remained significantly reduced compared to animals infused with norepinephrine alone (Figure 3.6E).

Propranolol infusion, which reduced blood pressure irrespective of sodium intake but failed to restore dietary sodium-evoked suppression of NCC activity in norepinephrine-infused rats, also failed to restore the suppression of NCC expression and phosphorylation during high salt intake (Figure 3.6A-B). Further, while β -adrenoceptor blockade restored suppression of SPAK expression during high salt intake in norepinephrine-infused rats, dietary sodium-evoked suppression

of the expression and phosphorylation of the NCC and OxSR1, as well as total WNK1 expression, remained impaired (Figure 3.6A-F).

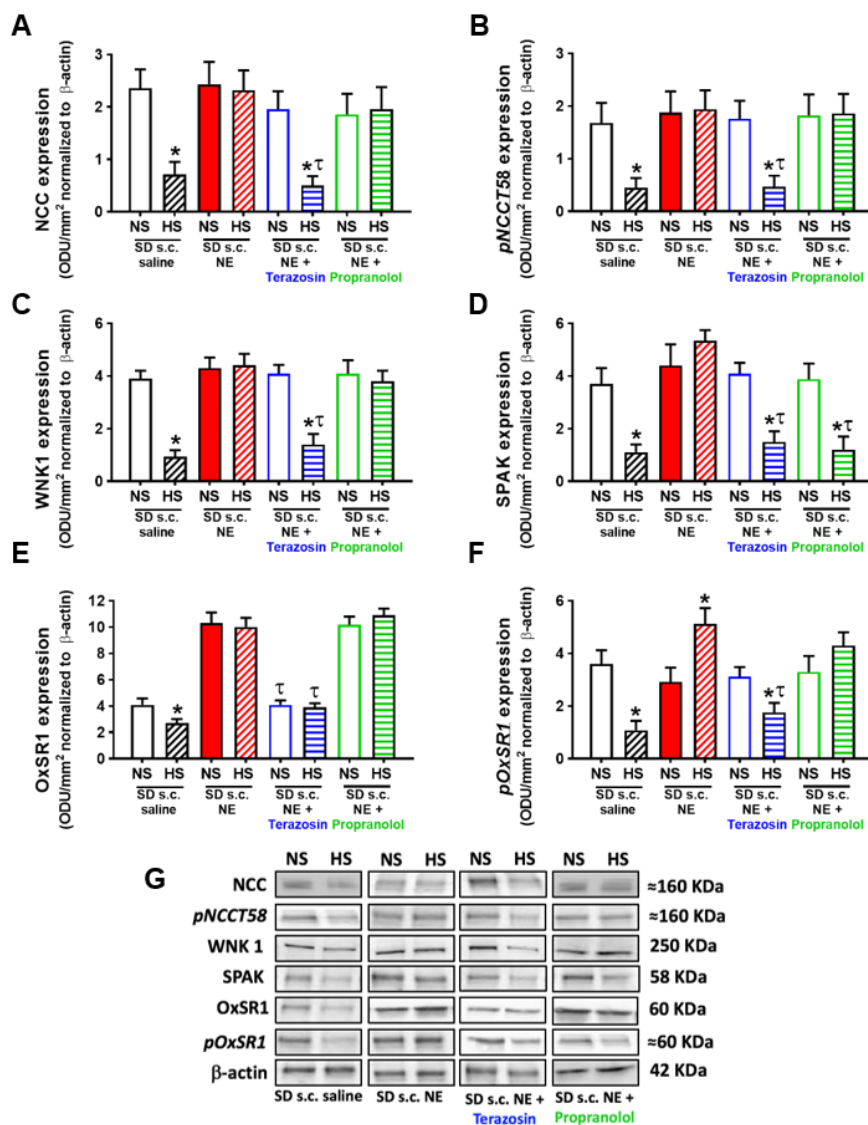


Figure 3.6 Impact of chronic α_1 - and β -adrenoceptor antagonism on the NCC and its regulatory kinases. Renal cortex (A) total NCC expression, (B) NCC phosphorylation (Thr58), (C) WNK1 expression, (D) SPAK expression, (E) OxSR1 expression, (F) OxSR1 phosphorylation, and (G) representative immunoblots from 3-month old male Sprague Dawley rats that received a subcutaneous (s.c.) infusion of saline, norepinephrine (NE), NE + terazosin, or NE + propranolol during a 21-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet. $n = 6/\text{group}$. * $P < 0.05$ vs. respective NS group, $\tau P < 0.05$ vs. respective NE-infused group.

Chronic α_1 -adrenoceptor antagonism reverses the salt sensitivity of blood pressure and restores dietary sodium-evoked NCC suppression in established norepinephrine-evoked salt-sensitive hypertension

A 42-day high salt diet had no impact on blood pressure and promoted the suppression of NCC activity in vivo in saline-infused rats (Figure 3.7). A 42-day norepinephrine infusion evoked hypertension but had no impact on NCC activity during normal salt intake and promoted the development of salt-sensitive hypertension accompanied by a failure to suppress NCC activity during high salt intake (Figure 3.7). Terazosin infusion had no impact on blood pressure or NCC activity in norepinephrine-infused rats maintained on a normal salt diet (Figure 3.7). Critically, however, α_1 -adrenoceptor blockade reversed the salt-sensitive component of established norepinephrine-evoked salt-sensitive hypertension and restored suppression of NCC activity in rats maintained on a high salt diet (Figure 3.7).

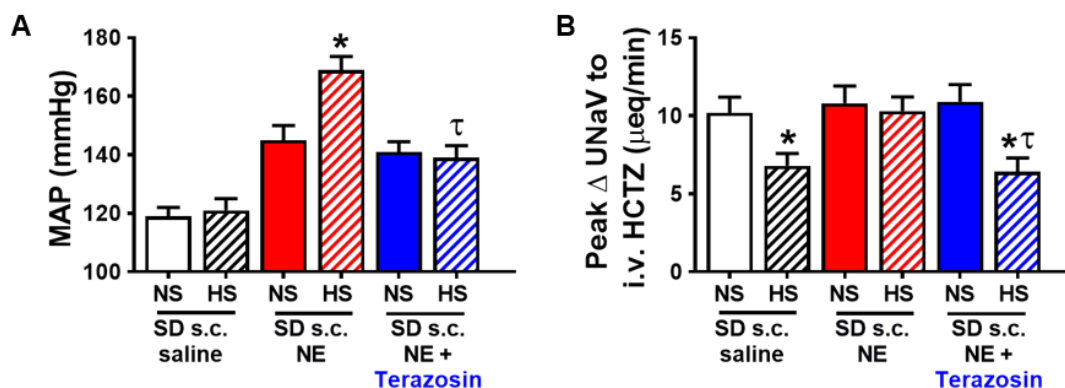


Figure 3.7 Impact of chronic α_1 -adrenoceptor antagonism on established norepinephrine-evoked salt-sensitive hypertension. (A) Mean arterial pressure (MAP; mmHg) and (B) peak natriuretic response (Δ UNaV; μ eq/min) to intravenous (i.v.) hydrochlorothiazide (HCTZ; 2mg/kg bolus, 2mg/kg hour infusion), in 3-month old male Sprague Dawley rats on a 42-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet that received a subcutaneous (s.c.) infusion of saline (42 days; black bars), norepinephrine (NE) alone (42 days; red bars), or NE alone for 21 days followed by NE + terazosin for 21 days (blue bars). $n = 6/\text{group}$. * $P < 0.05$ vs. respective NS group, $\tau P < 0.05$ vs. respective NE-infused group.

Discussion

The current studies were designed to delineate the adrenergic signaling pathways that promote NCC activity in norepinephrine-evoked salt-sensitive hypertension. Previous studies have demonstrated that exogenous norepinephrine drives increased NCC activity and salt-sensitive hypertension in Sprague Dawley rats (Walsh et al., 2016), which are classically salt resistant (Kapusta et al., 2012; Kapusta et al., 2013), and that salt-sensitive animal models exhibit excess sympathetic outflow during high salt intake that could contribute to inappropriate NCC activation (Mu et al., 2011; Terker et al., 2014). Critically, several studies have demonstrated that salt sensitivity in human subjects is characterized by a failure to suppress sympathetic tone during high salt intake

(Campese et al., 1982; Gill et al., 1988); however, the potential mechanistic link between sympathetic tone, NCC regulation, and salt sensitivity is incompletely understood.

Here, we confirmed our previous observation that norepinephrine infusion in the normotensive salt-resistant Sprague Dawley rat evokes the development of salt-sensitive hypertension (Walsh et al., 2016). Further, we used α_1 - and β -adrenoceptor antagonism to provide novel mechanistic insight into a selective α_1 -adrenoceptor-gated WNK1/OxSR1 signaling pathway that prevents the suppression of NCC expression, phosphorylation, and activity during high salt intake in norepinephrine-infused Sprague Dawley rats. Using chronic α_1 -antagonism, initiated at the start of high salt intake or after three weeks of high salt intake, we demonstrate that α_1 -adrenergic signaling pathways are required for both the development of salt sensitivity and the maintenance of established salt-sensitive hypertension in norepinephrine-infused rats. Together, our findings provide novel insight into the mechanisms through which elevated plasma norepinephrine concentration and impaired dietary sodium-evoked suppression of sympathetic tone may promote NCC-mediated sodium retention and salt-sensitive hypertension.

In the current studies, we observed that saline-infused Sprague Dawley rats maintain normotension during high salt intake and exhibit dietary sodium-evoked suppression of NCC mRNA and protein expression, phosphorylation, and physiological activity. In contrast, norepinephrine-infused animals exhibited salt-

sensitive hypertension characterized by impaired dietary sodium-evoked suppression of the NCC by multiple techniques, suggesting that the suppression of sympathetic tone during high salt intake is critical to NCC suppression and salt resistance. These observations are consistent with previous studies reporting dietary sodium-evoked suppression of the NCC in normotensive Sprague Dawley rats (Yang et al., 2008; Walsh et al., 2016) and linking sympathetic stimulation of the NCC with salt-sensitive hypertension in Sprague Dawley rats and other rodent models (Mu et al., 2011; Terker et al., 2014; Walsh et al., 2016).

During normal salt intake, norepinephrine infusion had no impact on NCC activity or expression but resulted in an increase in blood pressure that could be attributed to the effects of norepinephrine in other systems, including the vasculature, that are beyond the scope of these studies. Although studies in mice have reported a norepinephrine-evoked increase in NCC expression during normal salt intake (Mu et al., 2011; Terker et al., 2014), our finding that norepinephrine alone does not alter NCC activity or expression is consistent with previous studies in Sprague Dawley rats (Sonalker et al., 2008; Walsh et al., 2016) and suggests that there may be species differences in the adrenergic regulation of the NCC.

Building upon these previous studies that largely support our *in vivo* findings, we assessed protein expression of the kinase network known to influence NCC activity. A number of studies support with no lysine kinases WNK1 and WNK4 as regulators of NCC activity. WNK1 stimulates NCC activity via SPAK and OxSR1 (Moriguchi et al., 2005), while WNK4 decreases NCC activity by preventing its

translocation to the plasma membrane (Subramanya et al., 2009) or increases NCC activity via SPAK and OxSR1 (San-Cristobal et al., 2009). An initial study specifically assessing adrenergic regulation of the NCC in the context of salt-sensitive hypertension suggested that norepinephrine activates a β 2-adrenergic pathway involving WNK4, but not WNK1 (Mu et al., 2011). Two subsequent studies failed to replicate the role of WNK4 but did not further assess WNK1 (Uchida et al., 2012; Terker et al., 2014), and as such the potential role of WNK1 in norepinephrine-mediated NCC activation remains largely unaddressed. In the current studies, we observed that total WNK1, OxSR1, and SPAK protein expression was suppressed during high salt intake in salt-resistant saline-infused rats but not in norepinephrine-infused rats exhibiting salt-sensitive hypertension. These changes parallel the impairments in dietary sodium-evoked suppression of NCC activity, expression, and phosphorylation that likely contribute to salt-sensitive hypertension in norepinephrine-infused animals, providing evidence for a novel role of WNK1 in norepinephrine-mediated NCC regulation in this particular animal model of salt sensitivity.

Importantly, phosphorylation of OxSR1 – which occurs downstream of WNK1 and reflects activation of OxSR1 (Moriguchi et al., 2005), leading to NCC phosphorylation and activation (Richardson et al., 2008) – is suppressed during high salt intake in saline-infused rats but increases during high salt intake in norepinephrine-infused animals. While previous studies have suggested a critical role for SPAK in NCC regulation, with a relatively minor contribution of OxSR1, our

finding is consistent with a previous *in vivo* study in which kidney-specific deletion of OxSR1 severely attenuated acute norepinephrine-evoked NCC phosphorylation assessed after 30 minutes of norepinephrine stimulation (Terker et al., 2014). Our *in vivo* assessment of physiological NCC activity builds upon this previous study, which relied upon NCC phosphorylation as an established marker of NCC activity.

The activity of the NCC can be enhanced via phosphorylation at a number of key residues. In the current studies, we observed an increase in phosphorylation at Thr58, which can occur downstream of OxSR1 and SPAK (Moriguchi et al., 2005; Vitari et al., 2005; Richardson et al., 2008) and both stabilizes the NCC in the plasma membrane and increases its intrinsic activity (Yang et al., 2013). Previous studies have demonstrated that mutations that prevent phosphorylation at Thr58, which corresponds to Thr60 in the human NCC protein, also prevent phosphorylation at other residues and significantly reduce NCC activity (Richardson et al., 2008; Glover et al., 2009). This evidence suggests that the observed increase in Thr58 phosphorylation contributes significantly to the increase in NCC activity in the current studies. However, the potential role of phosphorylation at other residues relevant to NCC activation, and the role of other posttranslational modifications including ubiquitylation, which reduces NCC activity (Arroyo et al., 2011) and may be prevented by OxSR1- and SPAK-mediated NCC phosphorylation (Hossain Khan et al., 2012), requires further studies beyond the scope of this investigation. While the mechanisms governing the observed alterations in NCC expression are also unclear, several recent studies have

demonstrated that both SPAK and OxSR1 can modulate NCC protein expression (McCormick et al., 2011; Ferdaus et al., 2016).

To investigate the differential roles of α_1 - and β -adrenoceptors in norepinephrine-evoked salt-sensitive hypertension, we assessed the impact of chronic adrenoceptor antagonism on blood pressure, NCC activity, and the WNK1 signaling cascade observed in norepinephrine-infused rats. Antagonism of α_1 - or β -adrenoceptors had no impact on blood pressure or NCC activity in saline-infused rats regardless of dietary sodium intake, suggesting that blood pressure and NCC activity are determined by other physiological factors under basal normotensive conditions. Interestingly, total OxSR1 expression was increased in norepinephrine-infused rats during normal salt intake, raising the possibility that the established impact of OxSR1 on other sodium transporters not studied in the present work, such as NKCC2 (Moriguchi et al., 2005), could contribute to increased blood pressure in these animals.

In norepinephrine-infused animals placed on a high salt diet, the development of the salt-sensitive component of hypertension was abolished by α_1 blockade, which may reflect the restoration of dietary sodium-evoked suppression of NCC activity, expression, and phosphorylation. In contrast, antagonism of β -adrenoceptors attenuated norepinephrine-evoked hypertension independently of dietary sodium intake but failed to restore dietary sodium-evoked suppression of NCC activity, expression, and phosphorylation. These data suggest a potential critical and selective role for α_1 -adrenergic signaling pathways in norepinephrine-

evoked NCC dysregulation and salt-sensitive hypertension that conflicts somewhat with previous studies. In a study of norepinephrine-infused C57Bl/6J mice, antagonism of β -adrenoceptors, but not α_1 -adrenoceptors, attenuated norepinephrine-induced downregulation of WNK4 mRNA and upregulation of NCC protein expression (Mu et al., 2011). The reasons for the discrepancies between these observations, which have not been replicated (Uchida et al., 2012; Terker et al., 2014), and our own, which suggest a selective role for α_1 -adrenoceptors, are unclear but may include species differences. Supporting this reasoning, the same study also reported that norepinephrine infusion evoked an increase in NCC expression and phosphorylation during normal salt intake in mice, which we did not observe in Sprague Dawley rats in our current and previously published studies (Mu et al., 2011; Walsh et al., 2016). Interestingly, our observation of a role for α_1 -adrenoceptor signaling is supported to a degree by a subsequent study in C57Bl/6J mice, which failed to replicate a role of WNK4 in norepinephrine-evoked upregulation of NCC protein expression during high salt intake but confirmed a role of β -adrenoceptors and provided evidence that α_1 -adrenoceptors play a synergistic, albeit minor, part in acute NCC regulation (Terker et al., 2014).

Further supporting our *in vivo* observation of a role for α_1 -adrenoceptor signaling, α_{1D} -adrenoceptor mRNA expression was increased by norepinephrine infusion and remained elevated in norepinephrine-infused rats during high salt intake. Although the role of the α_{1D} -adrenoceptor in the kidney is incompletely characterized, salt-sensitive hypertension is attenuated in α_{1D} -adrenoceptor

knockout mice (Tanoue et al., 2002) and it is possible that increased signaling via α_{1D} -adrenoceptors contributes to salt sensitivity in our model. Interestingly, mRNA expression of α_{2B1} - and α_{2B2} -adrenoceptors is suppressed during high salt intake in saline-infused rats but not in norepinephrine-infused rats. Although the role of α_{2B} -receptor signaling in renal sodium handling is also poorly understood, a role for dietary sodium-evoked suppression of renal α_2 -adrenoceptor signaling in the sympathoinhibitory reno-renal reflex that promotes sodium homeostasis in salt-resistant models has been proposed (Kopp et al., 2011b). It is therefore possible that the failure to suppress α_{2B1} - and α_{2B2} -adrenoceptor mRNA during high salt intake in norepinephrine-infused animals is functionally relevant. Similarly to α_2 -adrenoceptors, β_2 -adrenoceptor mRNA expression is reduced during high salt intake in saline-infused rats; however, β_2 -adrenoceptor mRNA expression is reduced to a similar degree in norepinephrine-infused rats irrespective of sodium intake, supporting a specific role for α -adrenoceptor signaling in our model of salt sensitivity.

The important role of α_1 -adrenoceptor signaling supported by our current studies may reflect species differences but may also reflect differences in experimental paradigm, as the minor role of α_1 -adrenoceptors in mice was only assessed as the change in NCC phosphorylation following 30 minutes of α_1 - and/or β -adrenoceptor stimulation (Terker et al., 2014). In comparison, the current studies evaluated NCC activity, mRNA and protein expression, and phosphorylation following 3 weeks of norepinephrine co-infused with an α_1 - or β -antagonist during

both normal and high salt intake, and selective adrenoceptor blockade was validated in all animals as the loss of cardiovascular response to the respective adrenoceptor agonist. It is possible that the selective role of α_1 -adrenoceptor signaling is relevant in the longer term, while β -adrenoceptors play a more important role in the short term, although the role of β -adrenoceptors was initially proposed in a chronic study (Mu et al., 2011). A long-term regulatory role of α_1 -adrenoceptors is supported by our observation that α_1 -antagonism initiated following 3 weeks of high salt intake in norepinephrine-infused rats, a timepoint at which salt-sensitive hypertension is already established, attenuates the salt-sensitive component of hypertension and restores suppression of NCC activity. Importantly, while chronic norepinephrine infusion evokes salt-sensitive hypertension in mice, blood pressure is only mildly elevated by chronic norepinephrine infusion during normal salt intake (Terker et al., 2014). The acute adrenoceptor stimulation studies were performed in mice on a normal salt diet and blood pressure was not measured (Terker et al., 2014), raising the possibility that a role for α_1 -adrenoceptors in NCC regulation exists in mice but is limited to the context of salt-sensitive hypertension, as observed in the current studies. It is also worth considering the contrasting approaches of isolated adrenoceptor stimulation using pharmacological agonists versus adrenoceptor antagonism during norepinephrine infusion, which may better reflect the balance of α -/ β -adrenoceptor activation occurring during increased sympathetic outflow – which has been

observed during high salt intake in salt sensitive individuals (Campese et al., 1982; Gill et al., 1988).

While these discrepancies between species and potential acute versus chronic NCC regulation require further clarification, our observation of a selective role of α_1 -antagonism, which abolishes salt sensitivity and restores dietary sodium-evoked suppression of NCC activity, expression, and phosphorylation, is supported by our finding that antagonism of α_1 -adrenoceptors, but not β -adrenoceptors, restores dietary sodium-evoked suppression of WNK1 and phosphorylated OxSR1 and reduces OxSR1 expression in norepinephrine-infused rats regardless of dietary sodium intake. Interestingly, β -adrenoceptor antagonism does restore dietary sodium-evoked suppression of SPAK in norepinephrine-infused animals. Although this is not associated with restoration of appropriate NCC regulation, suggesting a specific role for an α_1 -adrenoceptor-OxSR1 signaling cascade in NCC regulation, β -antagonism does abolish the salt-sensitive component of hypertension. It is possible that future studies of simultaneous α_1 - and β -adrenoceptor antagonism could reveal a synergistic role of the two receptors as previously reported (Terker et al., 2014). Further studies are also required to determine the mechanism by which β -adrenoceptor signaling contributes to salt sensitivity in the absence of altered NCC regulation, which may be mediated by SPAK.

The interpretation of our studies is limited in part by the systemic administration of norepinephrine, which can influence blood pressure via activation

of adrenoceptors outside of the kidney, including those in the vasculature and the heart. Future studies involving renal infusion of norepinephrine and kidney-specific lentiviral knockdown of NCC regulatory kinases would be particularly enlightening. It is also important to note that plasma norepinephrine levels observed in a previous study using norepinephrine in Sprague Dawley rats (Walsh et al., 2016) reached levels higher than those generally observed in human subjects (Campese et al., 1982; Gill et al., 1988). However, the authors noted that plasma norepinephrine content in norepinephrine-infused Spague Dawley rats was similar to that observed in Dahl Salt Sensitive rats (Walsh et al., 2016) and reasonably reflects the failure of dietary sodium-evoked sympathoinhibition that characterizes salt sensitivity in human subjects (Campese et al., 1982; Gill et al., 1988). This model may also be particularly relevant to the context of human aging, which is characterized by lifelong exposure to excessive dietary sodium intake (Powles et al., 2013), increased sympathetic tone (Esler et al., 1995), and a dramatic increase in the prevalence of hypertension (Muntner et al., 2018) as well as salt sensitivity (Luft et al., 1991). Given the apparent species specificity of our findings, further studies in other animal models of salt sensitivity could yield important information. In models including the Dahl Salt Sensitive rat, in which the development of salt sensitivity does not require exogenous norepinephrine, the specific role of renal norepinephrine could be elucidated using renal denervation.

Together, our findings suggest that norepinephrine drives the development of salt-sensitive hypertension in Sprague Dawley rats in part via an α_1 -

adrenoceptor gated, WNK1-SPAK/OxSR1 signaling pathway that promotes NCC activity. Further, we have demonstrated that targeting this pathway prevents the development of salt-sensitive hypertension and, critically, abolishes salt sensitivity in established hypertension, which more reasonably reflects the stage at which human hypertension is diagnosed and treated. The novel role of WNK1 suggested by our findings is especially important given the recent development of selective small-molecule WNK inhibitors that improve blood pressure in rodent models in which human WNKs are overexpressed (Yamada et al., 2017). Overall, our studies provide important insight into the adrenergic signaling cascade that promotes NCC-mediated sodium retention, which represents a group of mechanistically relevant potential therapeutic targets in the treatment of salt-sensitive hypertension.

CHAPTER FOUR: Role of the Afferent Renal Nerves in Sodium Homeostasis and Blood Pressure Regulation in Rats

Abstract

The afferent renal nerves influence natriuresis, sympathetic outflow, and blood pressure. However, the differential roles of the mechanosensitive and chemosensitive afferent renal nerves in the homeostatic responses to sodium challenges are incompletely understood. In this study, selective capsaicin-mediated afferent renal nerve ablation was used to establish the roles of the afferent renal nerves in the natriuretic, sympathoinhibitory, and blood pressure responses to 1) an acute isotonic volume expansion versus hypertonic saline infusion in Sprague Dawley rats and 2) chronic high salt intake in Sprague Dawley, Dahl Salt Resistant, and Dahl Salt Sensitive rats. Afferent renal nerve responsiveness during high salt intake was assessed via 1) in vivo manipulation of renal pelvic pressure and sodium concentration, and 2) ex vivo norepinephrine- and sodium-evoked renal pelvic substance P release. We demonstrate that the afferent renal nerves are required for maximal natriuretic and sympathoinhibitory responses to an acute volume expansion that increases renal pelvic pressure, but not a hypertonic saline infusion that does not alter renal pelvic pressure, in Sprague Dawley rats. In vivo afferent renal nerve responsiveness to elevated renal pelvic pressure (mechanoreceptor stimulus) and ex vivo responsiveness to norepinephrine increased during high salt intake in Sprague Dawley, but not Dahl Salt Sensitive, rats. In vivo and ex vivo afferent renal nerve responsiveness to

increased renal pelvic sodium concentration (chemoreceptor stimulus) was unaltered during high salt intake. Afferent renal nerve ablation evoked sympathetically-mediated salt-sensitive hypertension in Sprague Dawley and Dahl Salt Resistant rats and exacerbated Dahl Salt Sensitive rat hypertension. Together these findings indicate that the mechanosensitive afferent renal nerves contribute to the responses to acute and chronic challenges to sodium homeostasis.

Introduction

The sensory afferent renal nerves, comprised of mechanosensitive and chemosensitive fibers originating primarily from the renal pelvic wall, contribute to sympathoinhibitory and sympathoexcitatory reno-renal reflexes that modulate efferent renal sympathetic nerve activity, natriuresis, and blood pressure (Johns, 2014; Kopp, 2015). The sympathoinhibitory reno-renal reflex, likely driven by mechanosensitive afferent renal nerve fibers, has been implicated in the tonic regulation of renal sympathetic nerve activity in healthy, normotensive animal models (Kopp, 1993; Chien et al., 2000; Ma et al., 2002a; Johns & Abdulla, 2013; Johns, 2014; Kopp, 2015). In this reflex, activation of the afferent renal nerves results in the suppression of efferent renal sympathetic nerve activity, promoting a natriuretic response facilitating sodium homeostasis and normotension (Kopp, 1993; Johns & Abdulla, 2013; Johns, 2014; Kopp, 2015). In contrast, the sympathoexcitatory reno-renal reflex, which increases renal sympathetic outflow and sodium retention, is primarily mediated by chemosensitive afferent renal nerve

fibers that are activated in animal models of disease states, including heart failure and renal failure (Kopp, 1993; Johns & Abdulla, 2013; Johns, 2014; Barry & Johns, 2015; Kopp, 2015).

Salt sensitivity of blood pressure is characterized by an exaggerated pressor response to dietary sodium intake that independently predicts hypertension risk (Franco & Oparil, 2006; Appel et al., 2011). In salt-resistant individuals, dietary sodium evokes a sympathoinhibitory response that facilitates natriuresis and normotension (Lohmeier et al., 1999; Johns, 2014). In contrast, in most salt-sensitive individuals, sympathoexcitation promotes sodium retention and hypertension in response to dietary sodium intake (Brooks et al., 2005; Stocker et al., 2013). It was recently postulated that neurohumoral control of renal excretory function is the first line of defense against dietary sodium intake in the pathogenesis of hypertension (Evans & Bie, 2016); however, the role of the afferent renal nerves in this process is unknown. A potential role for afferent renal nerve regulation of natriuresis and blood pressure is suggested by increased afferent renal nerve activity during high dietary sodium intake in the salt-resistant Sprague Dawley rat (Kopp et al., 2006; Kopp et al., 2009; Kopp et al., 2011b). The removal of all sensory afferent inputs from multiple end organs, including the afferent renal nerves, innervating multiple levels of the spinal cord, surgically via dorsal rhizotomy or pharmacologically by subcutaneous capsaicin evokes salt-sensitive hypertension in Sprague Dawley rats (Wang et al., 1998; Wang et al., 2001; Kopp et al., 2003). Significantly, direct electrical stimulation of the afferent

renal nerves, a non-specific stimulus that does not preferentially target mechanosensitive or chemosensitive afferent renal nerve terminals, activates hypothalamic paraventricular nucleus (PVN) parvocellular neurons and increases blood pressure (Solano-Flores et al., 1997; Xu et al., 2015). These data, likely reflecting activation of the sympathoexcitatory reno-renal reflex, raise the possibility that the PVN could also integrate the afferent and efferent arms of the afferent renal nerve-mediated sympathoinhibitory reno-renal reflex.

In the current study, we hypothesized that the afferent renal nerves contribute to the sympathoinhibitory reno-renal reflex that maintains sodium homeostasis and normotension during acute and chronic challenges to fluid and electrolyte homeostasis. To address this hypothesis, we used in vivo and ex vivo preparations to test the impact of dietary salt intake on afferent renal nerve reflexes. Selective afferent renal nerve ablation was used to investigate the role of the afferent renal nerves in the sympathoinhibitory, natriuretic, and blood pressure responses to 1) acute sodium challenges designed to differentially activate the mechanosensitive and chemosensitive afferent renal nerves and 2) a chronic high sodium diet in salt-resistant and salt-sensitive rat models.

Methods

All methods used in the studies of afferent renal nerve function in young adult animals in this chapter are briefly outlined in this section and described in further detail in Chapter 2. In brief, 3 month old male Sprague Dawley rats and

Dahl Salt Resistant rats were used as established models of salt resistance and Dahl Salt Sensitive rats were used as a model of salt sensitivity.

To test the role of the afferent renal nerves in the mechanosensitive and chemosensitive reno-renal reflexes in vivo, the cardiovascular, renal, and PVN neuronal responses to acute volume expansion and 1M NaCl infusion were assessed 10 days after selective afferent renal nerve ablation or a sham surgical procedure in conscious Sprague Dawley rats (Figure 4.1A). Glomerular filtration rate during volume expansion and 1M NaCl infusion was assessed in a subset of these animals (studies conducted during protocols shown in Figure 4.1A). In separate sets of anesthetized Sprague Dawley rats, renal pelvic pressure was measured during volume expansion and 1M NaCl infusion to validate volume expansion as a putative mechanoreceptor stimulus and 1M NaCl infusion as a selective chemoreceptor stimulus (Figure 4.1B).

To assess afferent renal nerve function in isolation, norepinephrine- and sodium-evoked substance P release was determined using an ex vivo renal pelvis assay in Sprague Dawley, Dahl Salt Resistant, and Dahl Salt Sensitive rats after a 21-day normal or high salt diet (Figure 4.1C). These studies were validated and extended in vivo via the direct manipulation of renal pelvic pressure and renal pelvic sodium concentration in anesthetized Sprague Dawley and Dahl Salt Sensitive rats following 21 days of normal or high salt intake (Figure 4.1D).

To investigate the contribution of the afferent renal nerves to sodium homeostasis and blood pressure regulation, radiotelemetry and metabolic balance

studies were conducted in Sprague Dawley, Dahl Salt Resistant, and Dahl Salt Sensitive rats that underwent selective afferent renal nerve ablation or a sham surgical protocol prior to a 21-day experimental normal salt diet (Sprague Dawley only) or high salt diet (all strains) (Figure 4.1E&F). The role of the afferent renal nerves in the regulation of sympathetic outflow (all strains) and the renin angiotensin aldosterone system (Sprague Dawley only) was assessed using tissues collected during these studies.

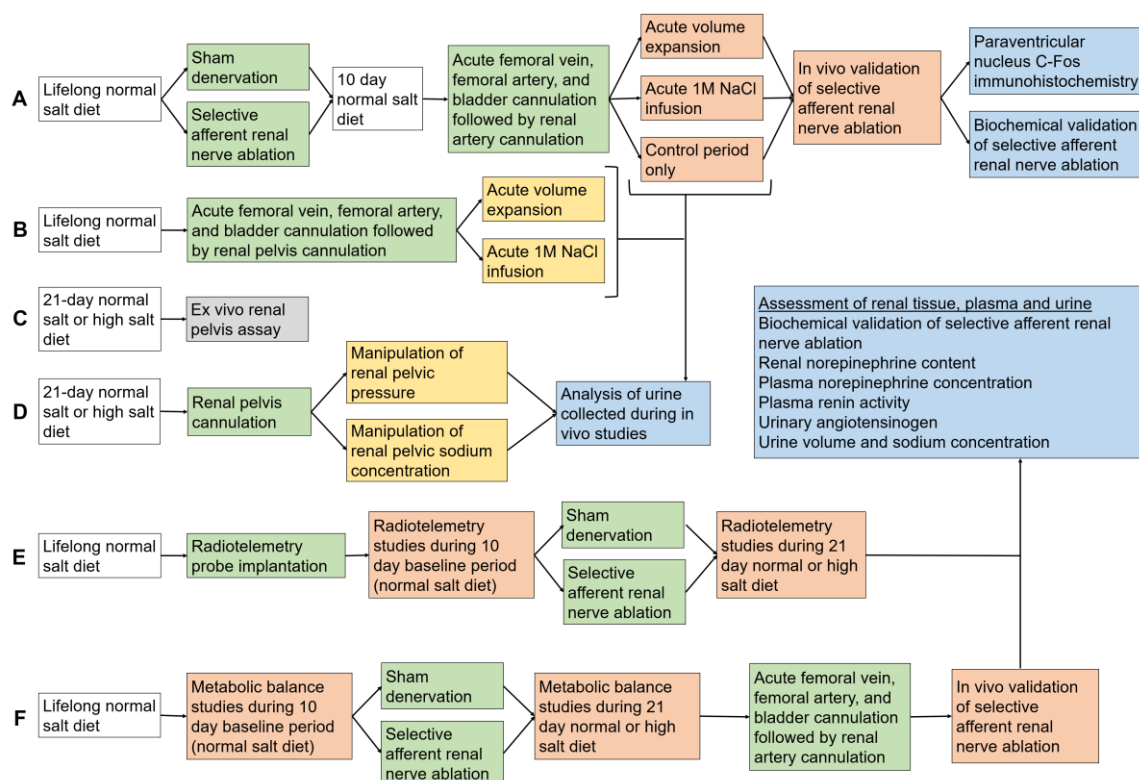


Figure 4.1. Overview of methods used in Chapter 4. All studies were performed using 3 month old male Sprague Dawley, Dahl Salt Resistant, and Dahl Salt Sensitive rats. White = dietary sodium assignments; green = surgical procedures; orange = in vivo studies in conscious animals; yellow = in vivo studies in anesthetized animals; grey = ex vivo studies; blue = analytical techniques. $n = 6/\text{group}$ unless specified in text. Detailed protocols are described in Chapter 2: General Methods.

Results

The afferent renal nerves modulate the cardiovascular, renal, and parvocellular PVN neuronal responses to an acute volume expansion

Conscious sham afferent renal denervated (ADNX) Sprague Dawley rats, in which surgery was performed but application of capsaicin was omitted to leave the afferent renal nerves intact, exhibited robust natriuretic and diuretic responses with a transient increase in free water clearance and no change in blood pressure, heart rate or renal hemodynamics (glomerular filtration rate and renal blood flow) during an acute volume expansion (Figures 4.2A&4.3A). This challenge evokes an increase in renal pelvic pressure in anesthetized naïve Sprague Dawley rats with intact afferent renal nerves (Figure 4.3B). Further, in sham ADNX rats an acute volume expansion resulted in increased Fos staining in all PVN parvocellular and magnocellular subnuclei (Figure 4.2B&C).

Selective ablation of the afferent renal nerves did not alter baseline cardiovascular or renal excretory parameters and had no impact on the baseline Fos staining in any region of the PVN (Figure 4.2A-C). In ADNX rats, the natriuretic, but not diuretic or renal hemodynamic response, to an acute volume expansion was significantly blunted and blood pressure was increased (Figure 4.2A). Similarly, volume expansion-evoked Fos staining of all PVN parvocellular neurons was attenuated in ADNX rats, while Fos activation in magnocellular PVN neurons remained intact (Figure 4.2B&C). The efficacy and selectivity of afferent renal nerve ablation was confirmed by a) the loss of blood pressure response to renal

artery bradykinin infusion, and b) the selective elimination of renal pelvic calcitonin gene-related peptide content, but not renal norepinephrine content (Figure 4.4).

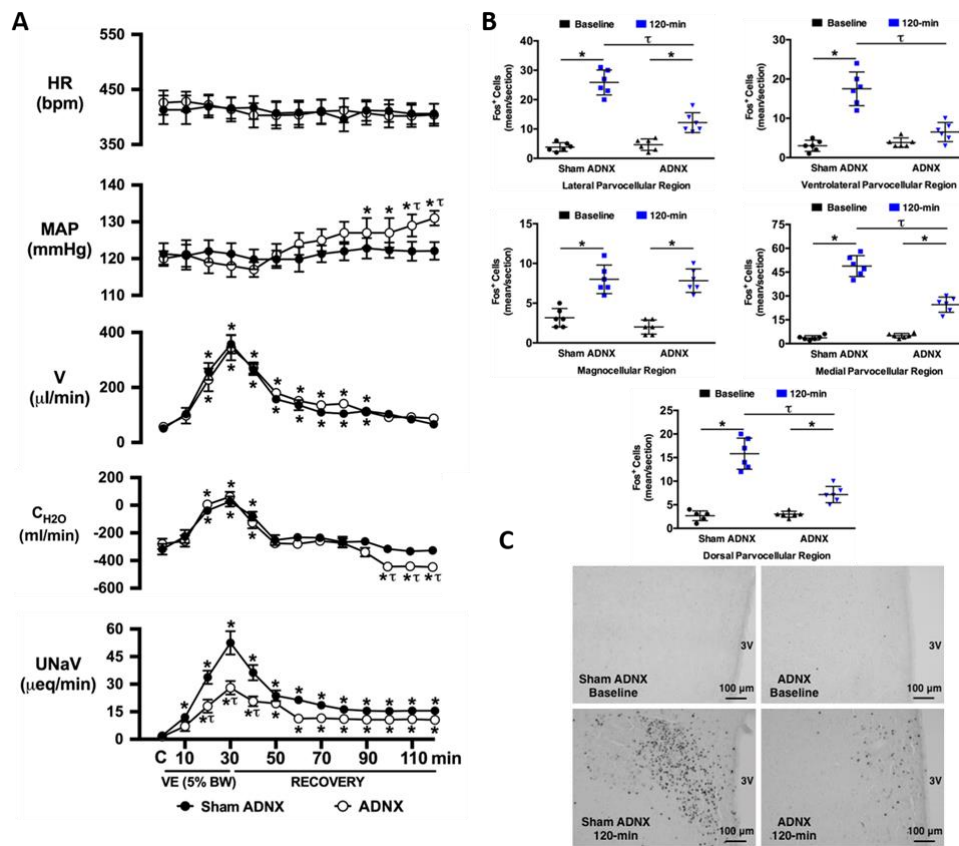
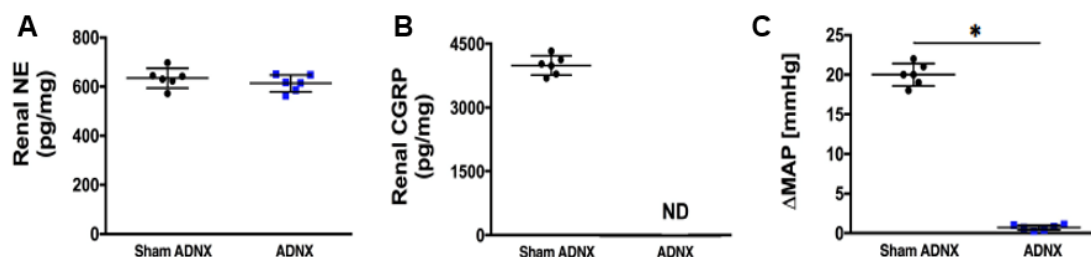
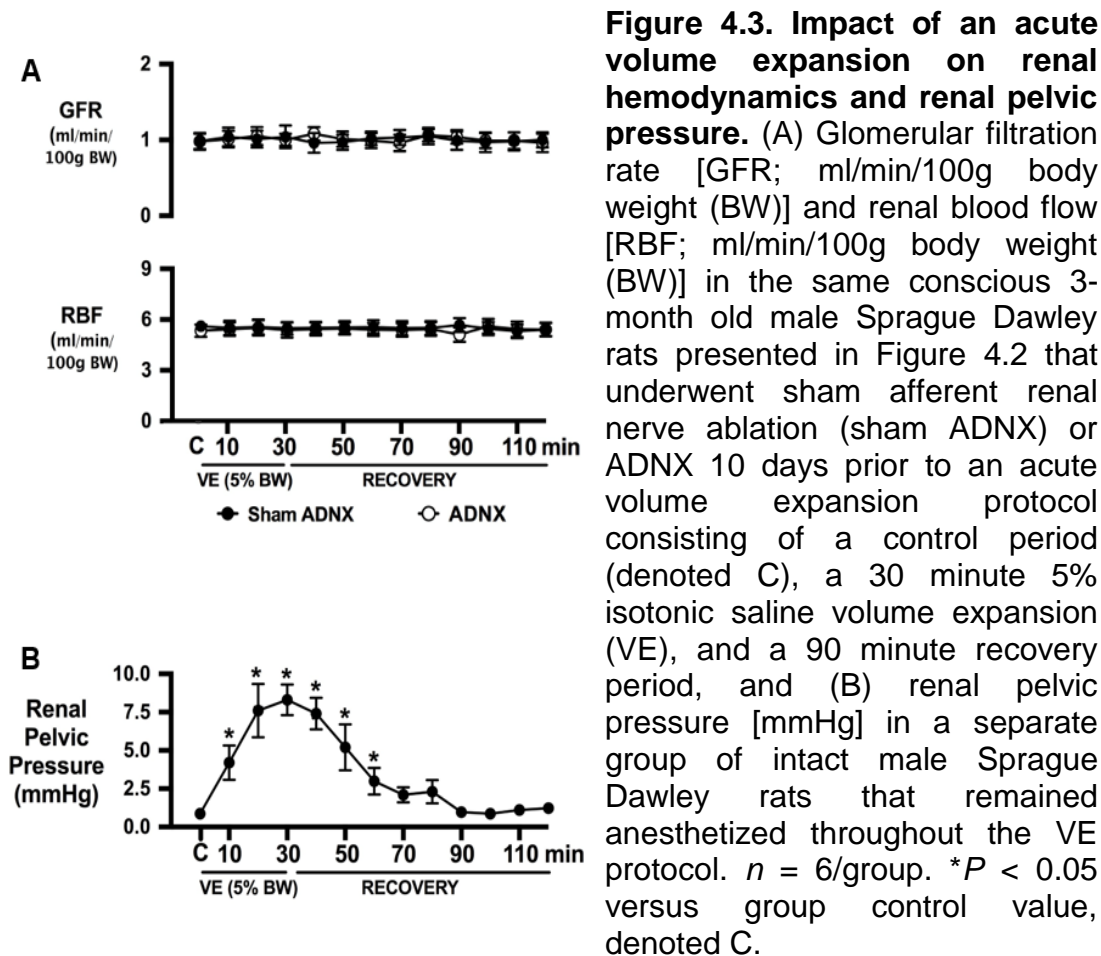


Figure 4.2. Effect of selective afferent renal nerve ablation on the cardiovascular, renal, and PVN neuronal responses to an acute volume expansion. (A) Cardiovascular and renal responses to a 30-minute 5% body weight isotonic saline volume expansion (VE) followed by a 90-minute recovery period in conscious male Sprague Dawley (SD) rats 10 days after a selective afferent renal nerve ablation (ADNX) or sham ADNX procedure, (B) neuronal activation (c-fos-positive cell count) in the lateral parvocellular, ventrolateral parvocellular, magnocellular, medial parvocellular, and dorsal parvocellular regions of the paraventricular nucleus (PVN) of the hypothalamus following VE or a 2-h surgical recovery and control period (baseline group) in conscious male SD rats 10 days after a sham or ADNX procedure and (C) representative images from level 2 of the PVN. HR = heart rate (bpm), MAP = mean arterial pressure (mmHg), V = urinary flow rate ($\mu\text{L}/\text{min}$), C_{H_2O} = free water clearance (ml/min), UNaV = urinary sodium excretion ($\mu\text{eq}/\text{min}$). $n = 6/\text{group}$. $*P < 0.05$ vs. group baseline; $\tau P < 0.05$ vs. respective sham ADNX value.



The afferent renal nerves are not required for the cardiovascular, renal, and PVN neuronal responses to an acute 1M NaCl infusion

In conscious sham ADNX Sprague Dawley rats, acute 1M NaCl infusion evoked a natriuretic response in the absence of diuresis, with a corresponding reduction in free water clearance, and had no impact on blood pressure, heart rate, glomerular filtration rate or renal blood flow (Figures 4.5A&4.6A). Further, an acute 1M NaCl infusion did not alter renal pelvic pressure in naïve intact anesthetized rats (Figure 4.6B). Fos staining was increased in all parvocellular and magnocellular PVN regions of sham ADNX rats following infusions of 1M NaCl (Figure 4.5B&C). Ablation of the afferent renal nerves did not alter cardiovascular, renal excretory or renal hemodynamic responses or PVN Fos staining during a 1M NaCl infusion (Figure 4.5A-C).

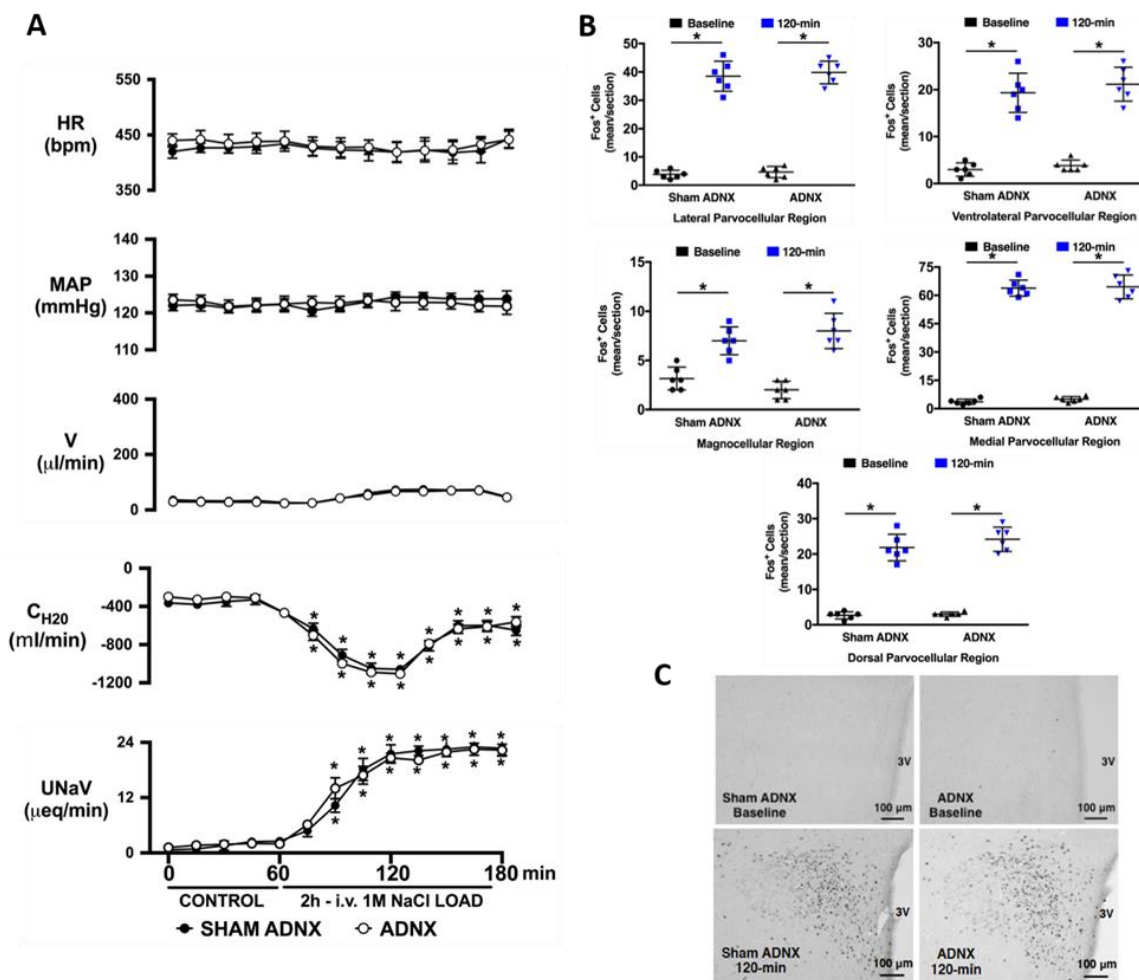


Figure 4.5. Effect of selective afferent renal nerve ablation on the cardiovascular, renal, and PVN neuronal responses to an acute 1M NaCl infusion. (A) Cardiovascular and renal responses to a 2-hour 1M NaCl infusion in conscious 3-month old male Sprague Dawley (SD) rats 10 days after a sham afferent renal nerve ablation (sham ADNX) or ADNX procedure, (B) neuronal activation (c-fos-positive cell count) in the lateral parvocellular, ventrolateral parvocellular, magnocellular, medial parvocellular, and dorsal parvocellular regions of the paraventricular nucleus (PVN) of the hypothalamus following a 2-h NaCl infusion or a 2-h surgical recovery and control period (baseline group) in conscious male SD rats 10 days after a sham ADNX or ADNX procedure and (C) representative images from level 2 of the PVN. HR = heart rate (bpm), MAP = mean arterial pressure (mmHg), V = urinary flow rate (μ L/min), C_{H_2O} = free water clearance (ml/min), UNaV = urinary sodium excretion (μ eq/min). $n = 6/\text{group}$. * $P < 0.05$ vs. group baseline.

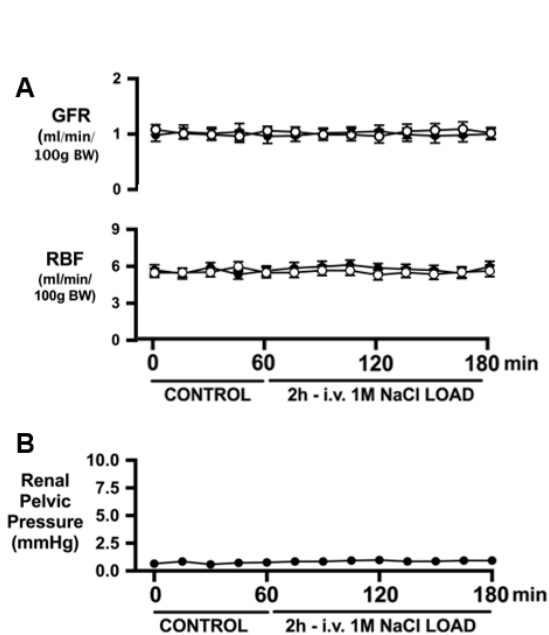


Figure 4.6. Impact of an acute 1M NaCl infusion on renal hemodynamics and renal pelvic pressure. (A) Glomerular filtration rate [GFR; ml/min/100g body weight (BW)] and renal blood flow [RBF; ml/min/100g body weight (BW)] in the same conscious male Sprague Dawley (SD) rats presented in Figure 4.5 that underwent sham afferent renal nerve ablation (sham ADNX) or ADNX 10 days prior to an acute 1M NaCl infusion protocol consisting of a 1-hour control period and a 2-hour 1M NaCl infusion (20 μ L/min), and (B) renal pelvic pressure [mmHg] in a separate group of intact male SD rats that remained anesthetized throughout the 1M NaCl infusion protocol. $n = 6$ /group. * $P < 0.05$ versus group control value, denoted C.

High salt intake increases afferent renal nerve responsiveness in salt-resistant but not salt-sensitive rat models

Ex vivo afferent renal nerve release of substance P in response to norepinephrine stimulation was comparable among Sprague Dawley, Dahl Salt Resistant, and Dahl Salt Sensitive rats on a normal salt diet (Figure 4.7A). A 21-day high salt diet enhanced ex vivo norepinephrine-evoked substance P release in salt-resistant Sprague Dawley and Dahl Salt Resistant rats, a response that was absent in Dahl Salt Sensitive rats (Figure 4.7A). In vivo, Sprague Dawley rats on a normal salt diet exhibit a natriuretic response to increased renal pelvic pressure (direct mechanoreceptor stimulus), and a 21-day high salt diet dramatically enhanced the natriuretic response to graded increases in renal pelvic pressure in

Sprague Dawley rats (Figure 4.7B-C). In contrast, increased renal pelvic pressure did not alter natriuresis in Dahl Salt Sensitive rats fed a normal salt diet and a dietary sodium-induced increase in natriuresis was only observed at the upper limit of the physiological range of increased renal pelvic pressure (Figure 4.7B-C).

Ex vivo afferent renal nerve release of substance P in response to 450mM NaCl, a chemoreceptor stimulus, was similar in Sprague Dawley, Dahl Salt Resistant, and Dahl Salt Sensitive rats on a normal salt diet and the response was unaffected by a 21 day high salt diet (Figure 4.7D). Further, manipulation of renal pelvic sodium concentration did not alter natriuresis in Sprague Dawley or Dahl Salt Sensitive rats regardless of dietary sodium intake (Figure 4.7E-F).

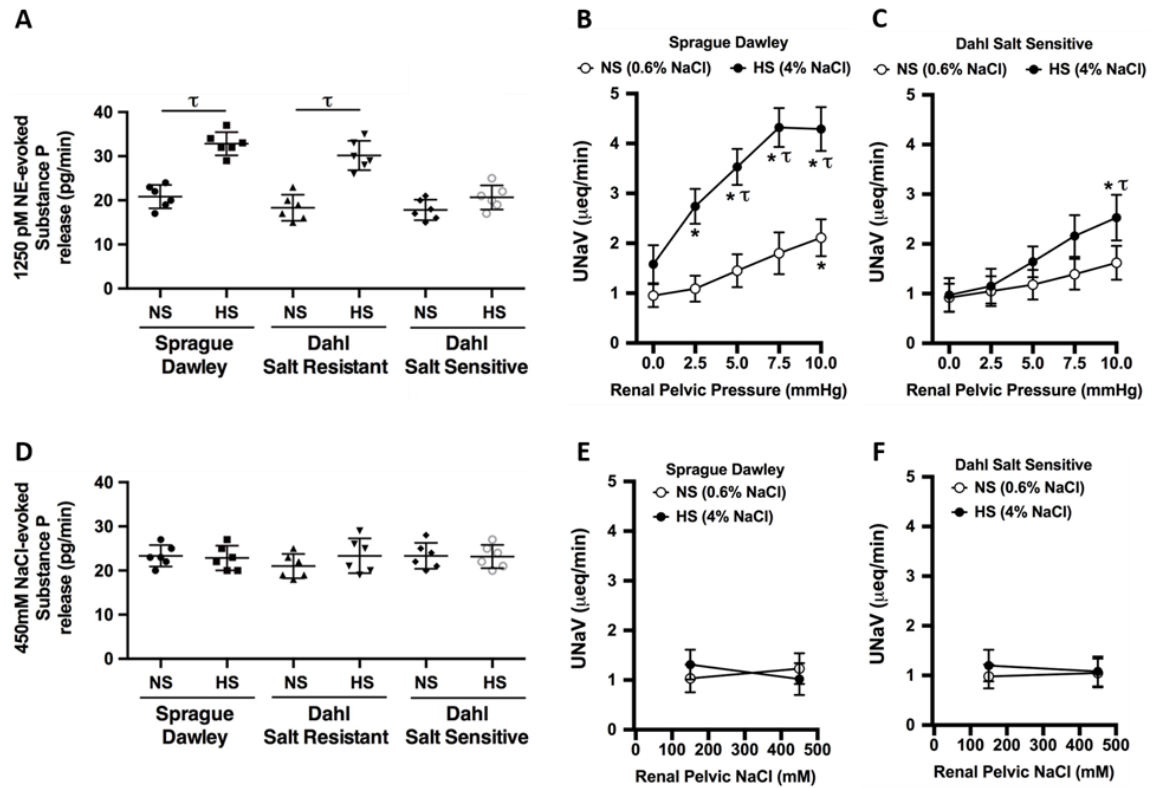


Figure 4.7. Effect of high salt intake on afferent renal nerve responsiveness.

(A) Ex vivo renal pelvis substance P release (pg/min) in response to 1250 pM norepinephrine (NE) on day 21 of a normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet in 3-month old male Sprague Dawley (SD), Dahl Salt Resistant (DSR), and Dahl Salt Sensitive (DSS) rats and urinary sodium excretion (UNaV) in response to graded increases in renal pelvic pressure in male (B) SD and (C) DSS rats following a 21-day NS or HS diet, (D) ex vivo renal pelvis substance P release in response to 450mM NaCl on day 21 of a NS or HS diet in male SD, DSR, and DSS rats and urinary sodium excretion in response to increased renal pelvic sodium concentration in male (E) SD and (F) DSS rats. $n = 6/\text{group}$. $*P < 0.05$ vs. group baseline, $\tau P < 0.05$ vs. respective NS group value.

The afferent renal nerves are required for Sprague Dawley and Dahl Salt

Resistant rats to maintain a salt-resistant phenotype

In Sprague Dawley rats fed a normal salt diet, sham ADNX and ADNX did not impact blood pressure, urine volume, or sodium balance (Figure 4.8A-C). Similarly, markers of renal and global sympathetic tone and renin-angiotensin system activity were similar in sham ADNX and ADNX Sprague Dawley rats on day 21 of normal salt intake (Figure 4.8D-G). A 21-day high salt diet did not alter blood pressure in sham ADNX Sprague Dawley and Dahl Salt Resistant rats, which possess intact afferent renal nerves (Figures 4.8A&4.9A). In contrast, Sprague Dawley and Dahl Salt Resistant rats that underwent ADNX to remove the influence of the afferent renal nerves immediately prior to high salt intake exhibited an increase in blood pressure during the first three days of high salt intake that was maintained for the remainder of the 21-day experimental period (Figures 4.8A&4.9A). These high salt-fed Sprague Dawley and Dahl Salt Resistant rats exhibited an increase in urine volume that was similar among sham and ADNX treatment groups (Figures 4.8B&4.9B). Further, high salt intake transiently increased daily sodium balance in sham ADNX Sprague Dawley and Dahl Salt Resistant rats, while this increase was significantly exacerbated in ADNX Sprague Dawley rats (Figures 4.8C&4.9C). The 21-day high salt diet evoked a reduction in plasma and renal norepinephrine content in sham ADNX Sprague Dawley rats and this reduction was abolished following ADNX (Figure 4.8D&E). In contrast, both sham ADNX and ADNX Sprague Dawley rats exhibited similar dietary sodium-

evoked reductions in plasma renin activity and urinary angiotensinogen (Fig 4.8F&G). Plasma and renal norepinephrine content were increased on day 21 of high salt intake in ADNX Dahl Salt Resistant rats compared to sham ADNX (Figure 4.9D&E). The efficacy of the ADNX procedure was confirmed in subgroups of rats via the absence of a pressor response to intrarenal bradykinin infusion and a significant reductions in renal pelvic calcitonin gene-related peptide content (Figure 4.10).

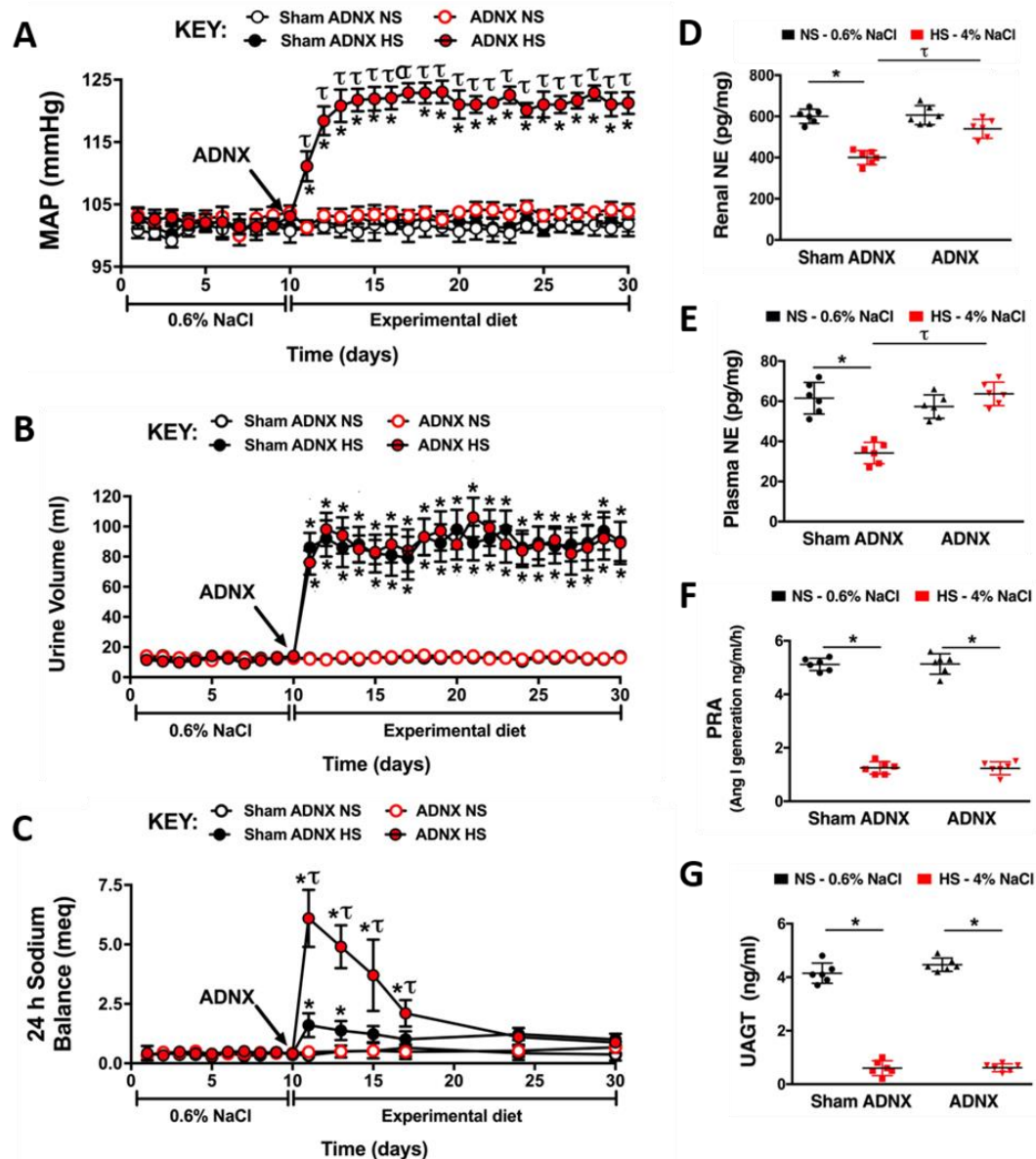


Figure 4.8. Effect of selective afferent renal nerve ablation on the cardiovascular, renal, and sympathetic responses to high salt intake in Sprague Dawley rats. Daily (A) mean arterial pressure (MAP; mmHg), (B) 24-hour urine volume (mL), and (C) 24-hour sodium balance (meq) in male Sprague Dawley rats that underwent sham afferent renal nerve ablation (sham ADNX) or ADNX immediately prior to a 21-day experimental normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet and (D) renal norepinephrine (NE) content (pg/mg), (E) plasma NE concentration (nmol/L), (F) plasma renin activity (PRA; angiotensin I [ang I] generation, ng/ml/h) and (G) urinary angiotensinogen (UAGT; ng/mL) on day 21 of NS or HS intake. $n = 6/\text{group}$. $*P < 0.05$ vs. baseline (NS intake); $\tau P < 0.05$ vs. respective sham ADNX group.

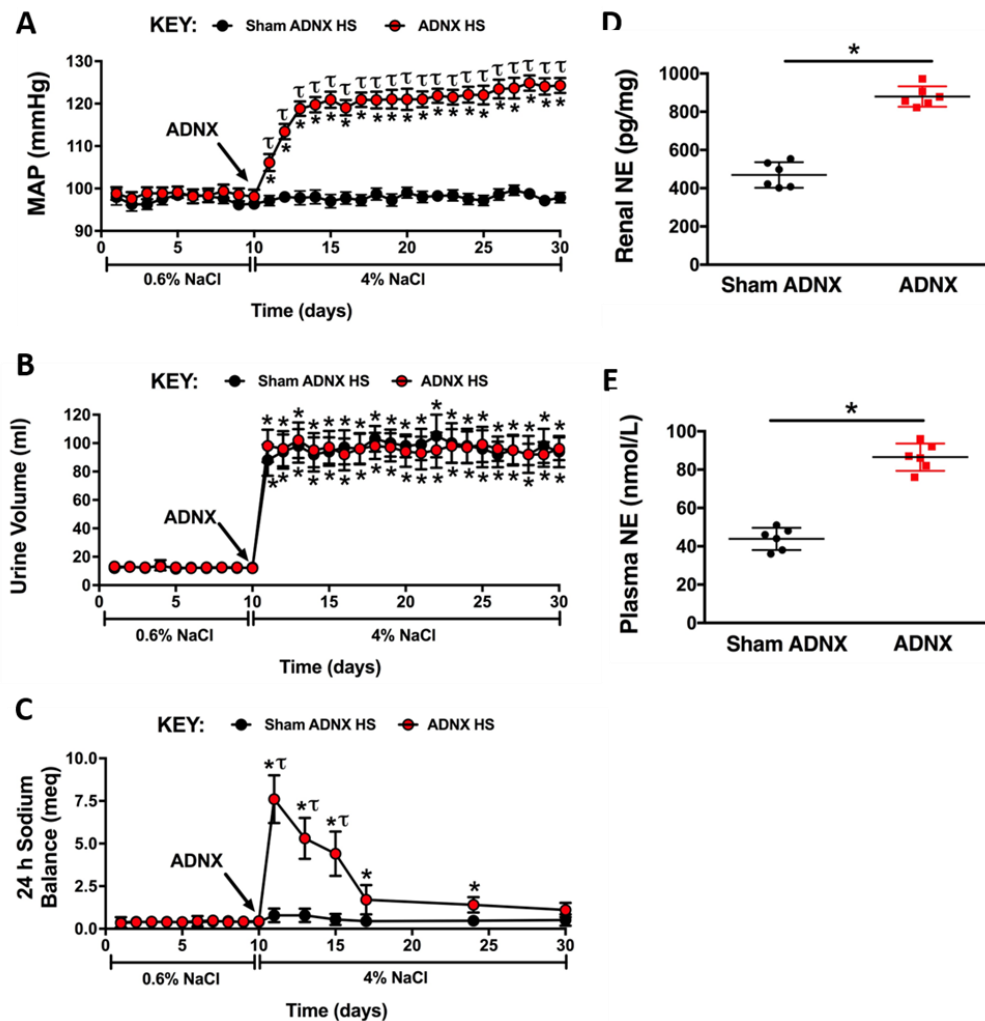


Figure 4.9. Effect of selective afferent renal nerve ablation on the cardiovascular, renal, and sympathetic responses to high salt intake in Dahl Salt Resistant rats. Daily (A) mean arterial pressure (MAP; mmHg), (B) 24-hour urine volume (mL), and (C) 24-hour sodium balance (meq) in 3-month old male Dahl Salt Resistant rats that underwent sham afferent renal nerve ablation (sham ADNX) or ADNX immediately prior to a 21-day experimental high salt diet (HS; 4% NaCl) and (D) renal norepinephrine (NE) content (pg/mg) and (E) plasma NE concentration (nmol/L) on day 21 of HS intake. $n = 6/\text{group}$. * $P < 0.05$ vs. baseline (normal salt intake; 0.6% NaCl); $\tau P < 0.05$ vs. sham ADNX.

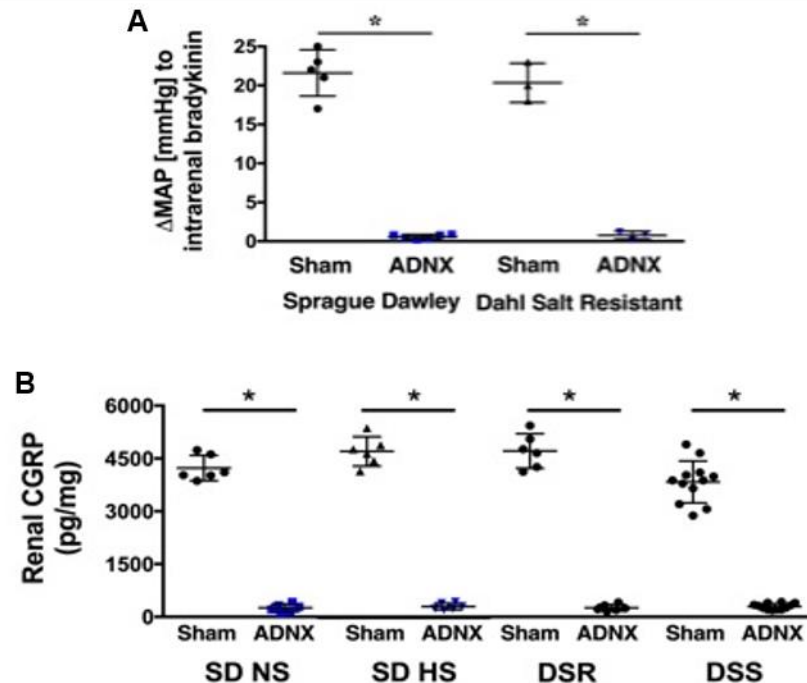


Figure 4.10. Validation of selective afferent renal nerve ablation following chronic dietary sodium intake studies. (A) Change in blood pressure (mmHg) in response to intrarenal bradykinin infusion in 3-month old male Sprague Dawley (SD) ($n = 5/\text{group}$) and Dahl Salt Resistant (DSR) rats ($n = 3/\text{group}$) and renal calcitonin gene-related peptide (CGRP) content (pg/mg) in SD ($n = 6/\text{group}$), DSR ($n = 6/\text{group}$), and Dahl Salt Sensitive (DSS) rats ($n = 12/\text{group}$) maintained on a normal salt (NS; 0.6% NaCl) or high salt diet (HS; 4% NaCl) following sham surgery or selective afferent renal nerve ablation (ADNX). * $P < 0.05$ vs. respective sham group.

The afferent renal nerves attenuate the development of Dahl Salt Sensitive hypertension

Sham ADNX Dahl Salt Sensitive rats placed on a high salt diet exhibited a significant increase in blood pressure by day 7 of high salt intake (Figure 4.11A). Afferent renal nerve ablation immediately prior to high salt intake exacerbated the increase in blood pressure (Figure 4.11A). Sham and ADNX Dahl Salt Sensitive rats exhibited similar increases in urine output during high salt intake (Figure

4.11B), with an increase in 24-hour sodium balance that was exacerbated in ADNX rats (Figure 4.11C). Consistent with increased sympathetic tone, plasma and renal norepinephrine content was increased in ADNX versus sham ADNX Dahl Salt Sensitive rats (Figure 4.11D&E). In contrast to the impact of ADNX conducted prior to high salt intake, ADNX performed after 16 days of high salt intake had no impact on the progression of Dahl Salt Sensitive hypertension (Figure 4.12A). Afferent renal nerve ablation was confirmed via a reduction of renal pelvic calcitonin gene-related peptide content in all rats (Figure 4.10B&4.12B).

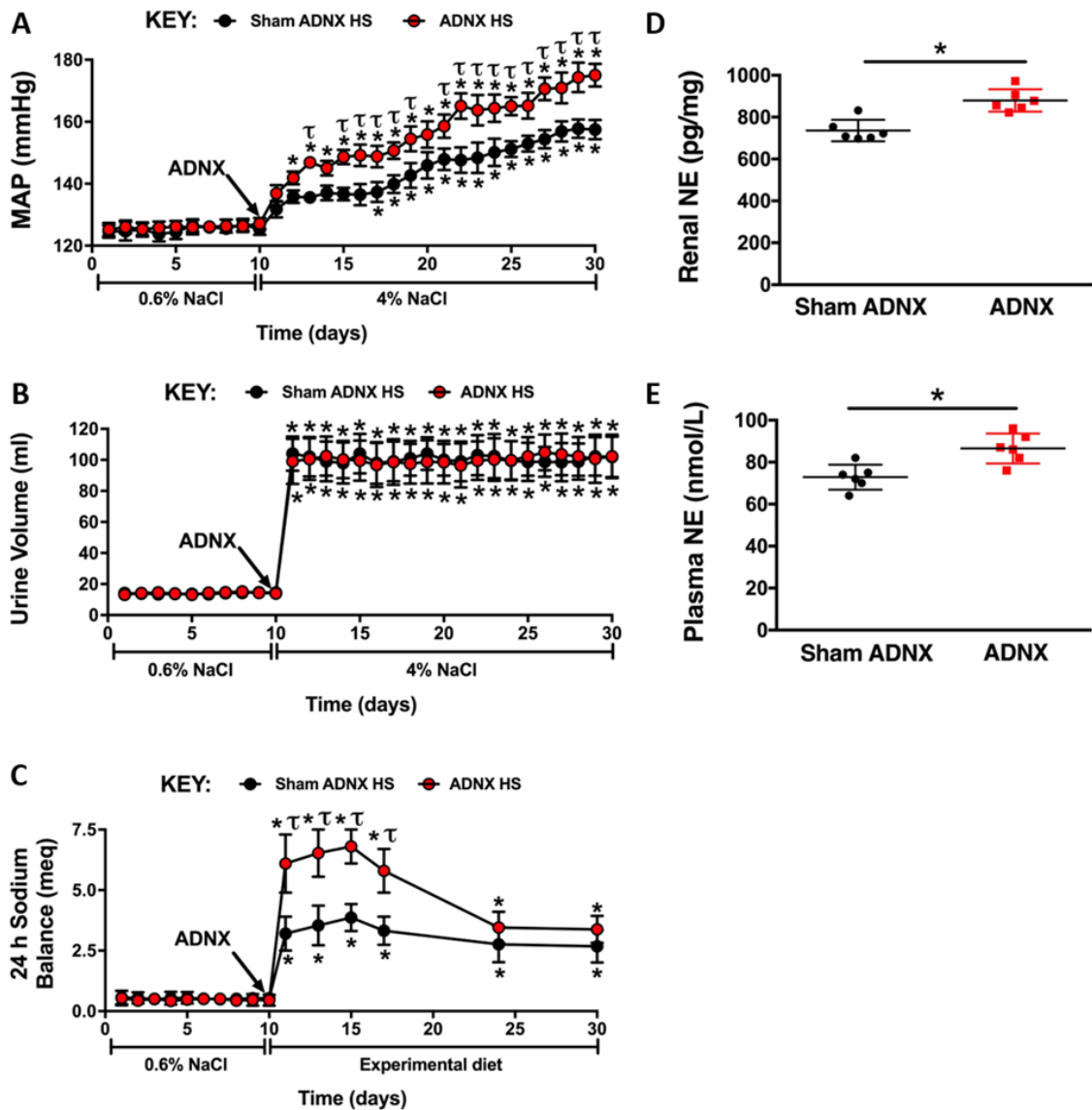


Figure 4.11. Effect of selective afferent renal nerve ablation on the cardiovascular, renal, and sympathetic responses to high salt intake in Dahl Salt Sensitive rats. Daily (A) mean arterial pressure (MAP; mmHg), (B) 24-hour urine volume (mL), and (C) 24-hour sodium balance (meq) in 3-month old male Dahl Salt Sensitive rats that underwent sham afferent renal nerve ablation (sham ADNX) or ADNX immediately prior to a 21-day experimental high salt (HS; 4% NaCl) diet and (D) renal norepinephrine (NE) content (pg/mg), and (E) plasma NE concentration (nmol/L) on day 21 of HS intake. $N = 6/\text{group}$. $*P < 0.05$ vs. baseline (normal salt intake; 0.6% NaCl); $\tau P < 0.05$ vs. sham ADNX.

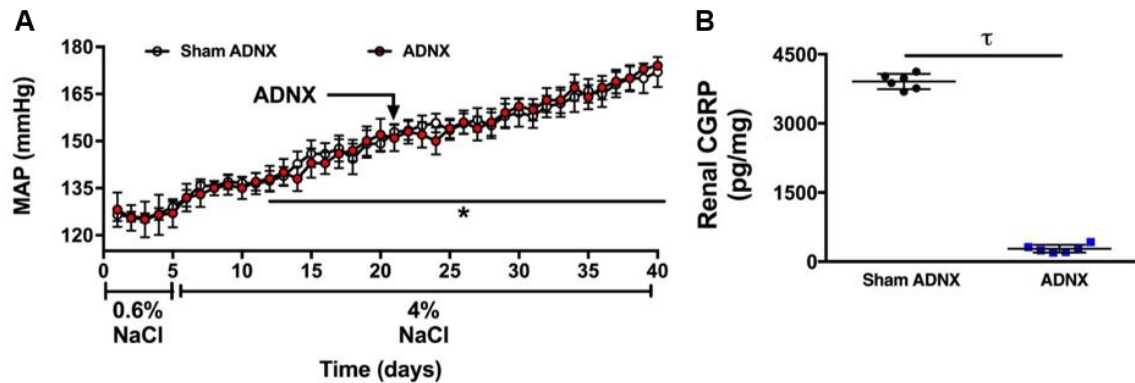


Figure 4.12. Effect of selective afferent renal nerve ablation on established Dahl Salt Sensitive hypertension. Daily (A) mean arterial pressure (MAP; mmHg) in 3-month old male Dahl Salt Sensitive rats that underwent sham afferent renal nerve ablation (sham ADNX) or ADNX after 16 days of high salt (4% NaCl) intake and (B) renal calcitonin gene-related peptide (CGRP) content (pg/mg) assessed on day 40. * $P < 0.05$ vs. baseline (normal salt intake; 0.6% NaCl), $\tau P < 0.05$ vs. sham ADNX.

Discussion

The current studies were designed to determine the role of the sensory afferent renal nerves in the regulation of renal sodium excretion and blood pressure during acute and chronic challenges to sodium homeostasis. Previous studies have identified a sympathoinhibitory reno-renal reflex mediated by the mechanosensitive afferent renal nerves that results in the suppression of efferent renal sympathetic outflow, natriuresis, and normotension (Kopp, 1993; Johns & Abdulla, 2013; Johns, 2014; Kopp, 2015). Further, non-selective global sensory afferent denervation evokes salt-sensitive hypertension in normally salt-resistant Sprague Dawley rats (Wang et al., 1998; Wang et al., 2001; Kopp et al., 2003). The current results extend these observations in three ways. First, they suggest a critical role of mechanosensitive sensory afferent renal nerve signaling in the

sympathoinhibitory natriuretic reno-renal reflex. Second, the results suggest that the PVN, previously implicated in the sympathoexcitatory reno-renal reflex (Xu et al., 2015), contributes to the sympathoinhibitory reno-renal reflex. Finally, our data extend previous studies that used non-selective global afferent denervation techniques that eliminate all sensory afferent inputs, including systemic capsaicin and dorsal rhizotomy, by employing selective ADNX to demonstrate that the afferent renal nerves are required for the maintenance of sodium balance and normotension during high salt intake in two models of salt resistance and attenuate dietary sodium-evoked hypertension in a salt-sensitive model.

Our initial studies into the potential actions and central sites of afferent renal nerve signal integration were conducted in conscious normotensive Sprague Dawley rats that underwent an acute volume expansion. There were technical limitations preventing the determination of renal pelvic pressure in conscious rats. We did, however, demonstrate that acute volume expansion increases renal pelvic pressure in anesthetized intact Sprague Dawley rats. Further, this same volume expansion produced a robust diuretic response in conscious sham Sprague Dawley rats. Together these results indicate that the acute volume expansion protocol used in the current study activates afferent renal nerve mechanoreceptors. This is consistent with previous work demonstrating that afferent renal nerve mechanoreceptors that selectively respond to increased renal pelvic pressure also respond to intravenous volume expansion (Chien et al., 2000). Previous studies have demonstrated an acute volume expansion activates

sympathoinhibitory parvocellular PVN neurons (Haselton et al., 1994; Randolph et al., 1998; Ng et al., 2004), resulting in the suppression of renal sympathetic outflow and a robust natriuretic response (Haselton et al., 1994; Ng et al., 2004). Consistent with these studies, in response to an acute volume expansion we observed an increase in Fos-positive cell bodies in all parvocellular PVN subnuclei and a strong natriuretic response with robust diuresis and increased free water clearance in sham ADNX Sprague Dawley rats. The lack of activation of PVN neurons in control baseline animals matches that reported by many laboratories, including our own prior study conducted in the same acute cardiovascular and renal experimental preparation used in these studies (Carmichael et al., 2016).

Selective afferent renal nerve ablation, validated biochemically and functionally, blunted volume expansion-evoked parvocellular, but not magnocellular, PVN neuronal activation and natriuresis. In ADNX rats, volume expansion evoked a delayed and mild increase in blood pressure. Interestingly, there was a time delay between the impaired natriuretic response, which was most marked during volume expansion, and the increase in blood pressure, which was observed only during the final 40 minutes of the 90-minute recovery period. This time delay could reflect the failure of initial mechanisms activated to accommodate volume expansion (e.g., vasodilation), but require further study. Overall, these data suggest that the mechanosensitive afferent renal nerve-mediated sympathoinhibitory reno-renal reflex may be critical to the maintenance of normotension via activation of sympathoinhibitory pathways. While Fos

immunostaining clearly suggests that ADNX reduces activation of PVN sympathoinhibitory neurons, this technique does possess limitations (Dampney & Horiuchi, 2003). For example, Fos staining does not always equate to neuron activation and additional evidence, including direct recording of both afferent and efferent renal nerve traffic, will be needed to confirm these PVN neurons are functionally activated and sympathoinhibitory in nature.

Collectively, these data suggest a specific role for the afferent renal nerves in volume expansion-evoked parvocellular PVN Fos induction and subsequent natriuresis. While the PVN has been previously implicated as a site of integration for the sympathoexcitatory reno-renal reflex (Xu et al., 2015), these findings represent the first evidence, to our knowledge, functionally implicating afferent renal nerve-mediated PVN neuronal activation in the mechanosensitive sympathoinhibitory reno-renal reflex. While our findings in ADNX rats support a specific role for the afferent renal nerves, volume expansion is also known to stimulate non-renal sensory afferent signaling pathways, including those mediated by atrial stretch, to activate parvocellular PVN neurons (Pyner et al., 2002) and suppress renal sympathetic outflow (Karim et al., 1972; Pyner et al., 2002). We speculate these non-renal pathways may contribute to the observed residual parvocellular PVN neuronal activation observed in some subnuclei following volume expansion in ADNX rats.

Conscious Sprague Dawley rats received a 1M NaCl infusion to assess the role of the chemosensitive afferent renal nerves, which respond to changes in the

chemical composition of fluid in the renal pelvis including alterations in sodium concentration (Wainford et al., 2013). The 1M NaCl infusion did not alter renal pelvic pressure in anesthetized Sprague Dawley rats. In sham ADNX Sprague Dawley rats, 1M NaCl infusion evoked robust natriuresis in the absence of diuresis, resulting in a profound reduction in free water clearance that reflects a highly concentrated urine product without a change in volume that could activate mechanoreceptors. Together, combined with our prior study that reported this infusion rate does not increase plasma sodium content (Wainford et al., 2013), these results indicate that this was a selective chemoreceptor stimulus. In the current study, selective removal of the afferent renal nerves does not alter the profound natriuresis or PVN parvocellular and magnocellular neuronal activation evoked by a 1M NaCl infusion. Our prior study reported that a 1M NaCl infusion suppresses central sympathetic outflow (Wainford et al., 2013), raising the possibility that the observed activated parvocellular PVN neurons could contribute to sympathoinhibition. Supporting our current observation of no role of the afferent renal nerves in 1M NaCl-evoked natriuresis is our previous finding that the sympathoinhibitory and natriuretic responses to 1M NaCl remain intact following bilateral renal denervation (Wainford et al., 2013). Together, these findings suggest that mechanisms independent of the sympathoinhibitory reno-renal reflexes, potentially involving other sodium/osmosensitive visceral afferents or circumventricular organs (Kinsman et al., 2017a; Kinsman et al., 2017b), mediate 1M NaCl-evoked sympathoinhibition and natriuresis. Therefore, we speculate that

the mechanosensitive sympathoinhibitory afferent renal nerve reno-reflex is a specific and rapid critical component of the neurohumoral control of renal excretory function proposed to act as the first line defense against salt and water retention and salt-sensitive hypertension (Evans & Bie, 2016).

A limitation of our 1M NaCl and volume expansion studies is that baseline blood pressure is mildly elevated compared to that observed in radiotelemetred rats. However, these baseline values are in line with previous studies performed in conscious animals in our laboratory in these rat models (Wainford & Kapusta, 2010; Kapusta et al., 2012; Wainford et al., 2013; Wainford et al., 2015; Carmichael et al., 2016; Walsh et al., 2016) and baseline pressures are identical between sham and ADNX treatment groups, strongly suggesting this mild elevation has not adversely impacted the data generated in this study. Additionally, it is not possible to selectively inhibit or ablate mechano- versus chemoreceptors to more directly assess their differential roles in the responses to acute and chronic challenges, and our afferent renal nerve ablation technique eliminates both afferent renal nerve subtypes. Given our careful characterization of the effects of a volume expansion and 1M NaCl infusion we believe it is plausible to evaluate these as preferentially mechano- versus chemosensitive stimuli.

To extend our *in vivo* data and more directly assess the afferent renal nerves in isolation, an *ex vivo* renal pelvis assay was used to test the effects of high salt intake on the afferent renal nerves in salt-resistant versus salt-sensitive rat models. Afferent renal nerve responsiveness, assessed as substance P release

in response to norepinephrine (a general afferent renal nerve stimulus that acts at α_1 -adrenoceptors to drive an increase in afferent renal nerve activity) (Kopp et al., 2007) is enhanced by a 21-day high salt diet in normotensive salt-resistant Sprague Dawley and Dahl Salt Resistant rats but not in hypertensive Dahl Salt Sensitive rats. This suggests that enhanced afferent renal nerve responsiveness during high salt intake may be protective and critical for salt resistance. A high salt diet has no impact on afferent renal nerve responsiveness to a direct chemoreceptor stimulus (450 mM NaCl – the upper range of urinary sodium content) in either salt-resistant or salt-sensitive rats. This suggests that enhanced responsiveness to norepinephrine may reflect a selective increase in the responsiveness of the mechanosensitive afferent renal nerves during high salt intake. Despite the lack of an enhanced response to a high salt diet, our data indicate that the afferent renal nerves in Dahl Salt Sensitive rats are fully responsive to direct afferent renal nerve stimuli and may be playing a functional role during normal salt intake. The results also indicate that a high salt diet significantly enhances the natriuretic response to increased renal pelvic pressure, which activates the mechanosensitive afferent renal nerves (Ma et al., 2002a; Ma et al., 2002b; Kopp et al., 2003), across a full physiological range in Sprague Dawley rats, while in Dahl Salt Sensitive rats renal pelvic pressure-evoked natriuresis is enhanced only near the upper physiological limit. Consistent with a specific role for the mechanosensitive afferent renal nerves in salt resistance, high salt intake had no impact on the natriuretic response to a chemoreceptor specific

stimulus (increased renal pelvic sodium concentration) (Ma et al., 2002a; Ma et al., 2002b) in Sprague Dawley or Dahl Salt Sensitive rats. Given the identical responses observed in the ex vivo renal pelvis preparation in Sprague Dawley and Dahl Salt Resistant rats, we elected to conduct anesthetized in vivo renal pelvic studies only in Sprague Dawley and Dahl Salt Sensitive rats. Our in vivo findings, in concert with our ex vivo data, suggests that the mechanosensitive sympathoinhibitory afferent renal nerve-mediated reno-renal reflex is selectively enhanced during high salt intake in salt-resistant rats and may play a protective role to maintain sodium balance and normotension.

Given our observation of an alteration in afferent renal nerve responsiveness during high salt intake, we extended our studies to delineate the impact of the afferent renal nerves on blood pressure, sodium balance, and sympathoinhibition during chronic dietary sodium loading, we performed ADNX immediately prior to high salt intake. In salt-resistant Sprague Dawley and Dahl Salt Resistant rats, removal of the afferent renal nerves abolished dietary sodium-evoked global and renal sympathoinhibition and induced salt-sensitive hypertension. Immediately upon high salt intake there was a rapid and transient increase in positive sodium balance while blood pressure remained elevated over the protocol duration, suggesting resetting of the set-point blood pressure to facilitate pressure-natriuresis and sodium homeostasis. The observed sodium retention and hypertension may reflect a loss of a protective mechanosensitive afferent renal nerve reflex. When challenged with high salt intake, all animals

dramatically increase urine output to approximately 90ml/day. This would increase renal pelvic pressure within a physiological range that would activate mechanosensitive afferent renal nerves.

Our observations contrast with prior work suggesting the afferent renal nerves have no impact on blood pressure during stepped increases in dietary sodium intake that concluded 7 weeks post-ADNX in Sprague Dawley rats (Foss et al., 2015). However, in the prior study (Foss et al., 2015), a) afferent renal nerve-independent adaptive mechanisms could have been activated during the two-week period between ADNX and high salt intake to facilitate sodium homeostasis and normotension – as demonstrated following total renal denervation (DiBona & Sawin, 1983) and b) afferent renal nerve functional re-innervation, which was detected biochemically as the return of 50% of renal pelvic calcitonin gene-related peptide content (Foss et al., 2015) and has been reported following total renal denervation as the return of substance P-immunoreactive fibers in the renal pelvic wall reaching 50% by 4 weeks and 100% by 9 weeks post-denervation (Mulder et al., 2013), could have occurred. To address the potential confounding effects of non-afferent renal nerve-mediated compensatory mechanisms and afferent renal nerve reinnervation, ADNX was performed immediately prior to the start of high salt intake, and absence of a pressor response to bradykinin and significantly reduced renal pelvic calcitonin gene-related peptide was confirmed 21-days post-ADNX to validate the functional efficacy of our approach. Critically, our data, validated in the Dahl Salt Resistant phenotype, align with prior studies implicating

removal of the afferent renal nerves as a causal mechanism in the salt sensitivity of blood pressure that develops following generalized sensory afferent denervation of all sensory afferent inputs via dorsal rhizotomy or subcutaneous capsaicin treatment in Sprague Dawley rats (Wang et al., 1998; Wang et al., 2001; Kopp et al., 2003). Our data in Dahl Salt Sensitive rats suggest that afferent renal nerve function changes during Dahl Salt Sensitive hypertension and high salt intake. Previous studies in which ADNX was performed after 3 or 9 weeks of high salt intake, when blood pressure is moderately and severely elevated, respectively, demonstrate that the afferent renal nerves do not play a role in the maintenance of early or late phase Dahl Salt Sensitive hypertension (Foss et al., 2016). In the current studies, we confirmed that removal of the afferent renal nerves after 16 days of high salt intake – approximately one week after a dietary sodium-evoked increase in blood pressure above baseline is first observed – has no impact on Dahl Salt Sensitive hypertension. In contrast, ADNX immediately prior to high salt intake, a time point at which our ex vivo data demonstrate the afferent renal nerves are functional and responsive, evokes a more severe blood pressure phenotype accompanied by exacerbated renal and global sympathoexcitation in Dahl Salt Sensitive rats, suggesting that the sympathoinhibitory afferent renal nerve-mediated reno-renal reflex has a potential minor role in countering the development of Dahl Salt Sensitive hypertension.

A limitation of the current studies is that the specific mechanisms underlying long-term sympathoinhibitory reno-renal reflex-mediated natriuresis, which

potentially include sympathetic modulation of a) renal sodium transporter activity, or b) the renin-angiotensin axis remain to be established. Additionally, the phenotype of PVN neurons regulated by mechanosensitive afferent renal nerves, their role in sympathoinhibitory reno-renal reflex signal integration during high salt intake, and the molecular and synaptic mechanisms driving afferent renal nerve-mediated PVN neuronal activation require clarification.

Our data suggest that the impact of the afferent renal nerves on blood pressure regulation depends on the model and stage of hypertension being investigated. Recent studies have demonstrated that Angiotensin II hypertension does not involve the afferent renal nerves (Foss et al., 2018). Significantly, there is an increase in resting afferent renal nerve activity in DOCA-salt hypertension (Banek et al., 2016), and selective ADNX reduces blood pressure in established DOCA-salt hypertension (Foss et al., 2015; Banek et al., 2016; Banek et al., 2018). These data suggest that excess afferent renal nerve activity contributes to the DOCA-salt model of hypertension and may reflect activation of the sympathoexcitatory reno-renal reflex that has been described in heart failure and ischemia (Zheng et al., 2018). In contrast, in the spontaneously hypertensive rat model, sympathoinhibitory afferent renal nerve reno-renal reflex activity is attenuated and renal sympathetic outflow is enhanced (Kopp et al., 1987). Thus, hypertension may involve impaired sympathoinhibitory reno-renal reflex activity, enhanced sympathoexcitatory reno-renal reflex activity, or a shift in the balance of the opposing reflexes. This variability in afferent renal nerve activity has been

proposed as a contributing factor to the mixed results of clinical trials employing bilateral renal nerve ablation to reduce blood pressure (Fudim et al., 2018).

The current findings indicate that there is a pivotal role of the mechanosensitive afferent renal nerve-mediated sympathoinhibitory reno-renal reflex in the homeostatic responses to acute sodium challenge in healthy normotensive Sprague Dawley rats. These data also provide evidence suggesting that the PVN can be regulated by mechanosensitive afferent renal nerve activity and may be a critical site of central integration of the acute sympathoinhibitory reno-renal reflex. Additionally, our studies, generated via *ex vivo* and *in vivo* studies, suggest that the afferent renal nerves play a protective role in the maintenance of salt resistance by contributing to dietary sodium-evoked sympathoinhibition and sodium balance. Collectively our data support a central role for the mechanosensitive afferent renal nerve-mediated sympathoinhibitory reno-renal reflex as a homeostatic mechanism required for the sympathoinhibitory and natriuretic responses to acute sodium challenge and as a first line of defense against the development of salt-sensitive hypertension in animal models. These data add to a growing body of literature indicating that variability in afferent renal nerve activity and function could influence the efficacy of bilateral renal nerve ablation as an anti-hypertensive intervention. We speculate that renal nerve ablation in hypertensive individuals in whom the sympathoinhibitory reno-renal reflex is intact would remove an essential natriuretic pathway, potentially exacerbating hypertension. The development of novel biomarkers or minimally-

invasive challenges (e.g., intravenous volume expansion) to test sympathoinhibitory reno-renal reflexes could guide patient selection for phenotypically targeted renal nerve ablation.

CHAPTER FIVE: Integrated Renal and Neural Mechanisms of Age-Related Hypertension

Abstract

Aging is associated with elevated sympathetic tone, increased prevalence of hypertension, and salt sensitivity of blood pressure. We hypothesized that impairments in the afferent renal nerve-mediated sympathoinhibitory reno-renal reflex contribute to sympathoexcitation and norepinephrine-evoked sodium chloride cotransporter (NCC) activity, promoting age-related hypertension. Using the Sprague Dawley rat as a model of normal aging, we assessed age-related changes in 1) the sympathoinhibitory, natriuretic, and blood pressure responses to an acute volume expansion (mechanoreceptor stimulus), acute hypertonic saline infusion (chemoreceptor stimulus), and chronic high salt intake, 2) ex vivo afferent renal nerve responsiveness, assessed as renal pelvic substance P release in response to norepinephrine (general stimulus) or sodium (chemoreceptor stimulus), and 3) NCC activity. Further, we used selective afferent renal nerve ablation and bilateral renal denervation to investigate the roles of the afferent renal nerves and efferent renal sympathetic nerves in age-related hypertension. We observed that aging rats develop salt-sensitive hypertension accompanied by 1) impaired homeostatic responses to volume expansion, but not hypertonic saline infusion, 2) a selective reduction in norepinephrine-evoked afferent renal nerve activity during normal salt intake and a failure to enhance afferent renal nerve responsiveness during high salt intake, and 3) increased basal sympathetic tone

and NCC activity and blunted dietary sodium-evoked suppression of sympathetic tone and NCC activity. Critically, while afferent renal nerve ablation did not alter sodium balance or blood pressure, bilateral renal denervation improved sodium balance and attenuated age-related hypertension. These findings suggest that aging is associated with impairments in the mechanosensitive afferent renal nerve-mediated sympathoinhibitory reno-renal reflex that may promote sympathoexcitation, driving NCC-mediated sodium retention and salt-sensitive hypertension.

Introduction

Aging is associated with increases in the prevalence of both salt sensitivity of blood pressure and hypertension (Luft et al., 1991; Muntner et al., 2018; Whelton et al., 2018). Sympathetic nervous system activity, which promotes renal sodium reabsorption and plays a role in the pathophysiology of both salt sensitivity and hypertension, also rises with age, and sympathetic tone correlates positively with blood pressure only in adults above the age of 40 (Esler et al., 1995; Esler et al., 2002; Narkiewicz et al., 2005). Studies in which bilateral renal denervation lowers blood pressure in hypertensive animal models and human patients highlight a pivotal role of the renal nerves in hypertension (Pires et al., 2015; Wainford et al., 2015; Gao et al., 2016; Townsend et al., 2017; Azizi et al., 2018; Kandzari et al., 2018). However, the mechanistic role of the renal nerves in hypertension has

primarily been investigated in animal models during young adulthood, and the contribution of the renal nerves to age-related hypertension is poorly understood.

The renal nerves are composed of sensory afferent renal nerves and efferent renal sympathetic nerves that comprise sympathoinhibitory and sympathoexcitatory reno-renal reflexes critical to sodium homeostasis and blood pressure regulation (Recordati et al., 1982; Katholi et al., 1983; Katholi et al., 1984; Ma et al., 2002a; Ma et al., 2002b; Kopp et al., 2003). In Chapter 4, we used selective afferent renal nerve ablation in healthy, normotensive young adult (3 month old) Sprague Dawley rats to delineate a specific role for the mechanosensitive afferent renal nerves in the acute sympathoinhibitory reno-renal reflex and provided evidence for a role of the paraventricular nucleus (PVN) in the central integration of this reflex. We demonstrated that young, salt-resistant Sprague Dawley rats exhibit enhanced afferent renal nerve activity during high salt intake, which likely reflects activation of the mechanosensitive afferent renal nerves. Further, extending studies in which that general sensory denervation using capsaicin or dorsal rhizotomy evokes salt-sensitive hypertension, we showed that the afferent renal nerves are required for the maintenance of salt resistance and may be a first line of defense against the salt sensitivity of blood pressure.

The sympathoinhibitory reno-renal reflex has been described primarily in young, normotensive animals, while the sympathoexcitatory reno-renal reflex appears to be more relevant to disease states that include renal ischemia and heart failure (Recordati et al., 1982; Rogenes, 1982; Zheng et al., 2018). Essential

hypertension could involve an impairment in sympathoinhibitory reno-renal reflex function, an exacerbation of sympathoexcitatory reno-reflex activity, or a shift in the balance of the opposing reflexes that promotes an increase in renal sympathetic outflow. Regardless of its origin, an increase in renal sympathetic outflow promotes sodium retention through its effects on the renal vasculature and several renal sodium transporters, including the sodium chloride cotransporter (NCC) (Walsh et al., 2016). During high salt intake, NCC activity and phosphorylation are suppressed in rats and mice, respectively (Mu et al., 2011; Terker et al., 2014; Walsh et al., 2016). Further, norepinephrine infusion increases NCC phosphorylation and expression in mice and prevents dietary sodium-evoked suppression of NCC activity and expression in rats, resulting in salt-sensitive hypertension (Mu et al., 2011; Terker et al., 2014; Walsh et al., 2016). While putative roles for both α_1 - and β -adrenoceptors and various downstream kinases in NCC regulation have been proposed (Mu et al., 2011; Terker et al., 2014), we delineated a specific α_1 -adrenoceptor-gated WNK1-OxSR1 signaling pathway activating the NCC in norepinephrine-infused young adult Sprague Dawley rats in Chapter 3.

In the current studies, we hypothesized that impairments in the afferent renal nerve-mediated sympathoinhibitory reno-renal reflex contribute to sympathoexcitation and norepinephrine-evoked NCC activity to promote age-related hypertension and salt sensitivity of blood pressure. To address this hypothesis, we used the Sprague Dawley rat as a model of normal aging to assess

the impact of age on the sympathoinhibitory, natriuretic, and blood pressure responses to an acute volume expansion (mechanoreceptor stimulus), an acute hypertonic saline infusion (chemoreceptor stimulus), or a chronic high salt diet. Further, we used an ex vivo renal pelvic preparation to investigate the impact of age on afferent renal nerve responsiveness to norepinephrine (general afferent renal nerve stimulus) or sodium (chemosensitive afferent renal nerve stimulus) during normal and high salt intake. Finally, we investigated the impact of aging on dietary sodium-evoked sympathoinhibition, NCC suppression, sodium homeostasis, and blood pressure regulation and used bilateral renal denervation and selective afferent renal nerve ablation to dissect the roles of the efferent renal sympathetic nerves and afferent renal nerves in these responses.

Methods

All methods used in the aging studies in this chapter are briefly outlined in this section and described in further detail in Chapter 2. For all studies, $n = 6/\text{group}$ unless otherwise specified in this section. In brief, male Sprague Dawley rats aged 3 months, 8 months, and 16 months were used as a model of normal aging. In observational studies, all age groups were used to assess the development of age-related changes (Figure 5.1A-D). In interventional studies, only 3 month old and 16 month old animals were used (Figure 5.1E-G).

To test the mechanosensitive and chemosensitive afferent renal nerve-mediated reno-renal reflexes in vivo, the natriuretic, diuretic, and PVN neuronal

responses (assessed as C-Fos-positive cell count) to acute volume expansion and 1M NaCl infusion were assessed in animals maintained on a normal salt diet (Figure 5.1A). To investigate afferent renal nerve function in isolation, rats were placed on a 21-day normal or high salt diet and norepinephrine and sodium-evoked substance P release were determined in an ex vivo renal pelvis assay (Figure 5.1B).

The activity and expression of the NCC and its regulatory kinases were assessed after a 21-day normal or high salt diet (Figure 5.1C&D). The contribution of α_1 -adrenoceptors to age-related hypertension and NCC regulation were investigated using a chronic subcutaneous infusion of terazosin (α_1 -adrenoceptor antagonist) (Figure 5.1E; $n = 3$ for 16-month old terazosin infusion group) and the contribution of increased NCC activity to age-related hypertension was assessed using a chronic subcutaneous infusion of hydrochlorothiazide (NCC antagonist) in animals maintained on a normal salt diet (Figure 5.1F; $n = 4$ for 16-month old hydrochlorothiazide group).

Global sympathetic tone following a 21-day normal or high salt diet was assessed as plasma norepinephrine content (Figure 5.1D). Renal sympathetic tone was determined in the same animals as renal norepinephrine content (Figure 5.1D). Vascular sympathetic tone was determined as the peak depressor response to ganglionic blockade in animals that underwent the assessment of NCC activity (Figure 5.1C). The contributions of the afferent renal nerves and renal sympathetic

nerves to age-related hypertension and NCC regulation were assessed using selective afferent renal nerve ablation or bilateral renal denervation.

To assess the potential contribution of renal damage and the renin angiotensin aldosterone system to age-related impairments in renal sodium handling and blood pressure regulation, blood, kidney tissue and urine samples were collected after a 21-day normal or high salt diet (Figure 5.1D). Measures of renal damage, including glomerulosclerosis and mesangial expansion, urinary kidney injury molecule-1 concentration, and proteinuria, and measures of renin angiotensin aldosterone system activity, including plasma aldosterone and plasma angiotensin II, were assessed in these samples (Figure 5.1D). Glomerular filtration rate was also assessed in vivo in a small subset of animals that underwent acute volume expansion or 1M NaCl infusion (not shown; studies conducted during protocols shown in Figure 5.1A; $n = 2/\text{group}$ due to very recent availability of MediBeacon glomerular filtration rate monitoring device).

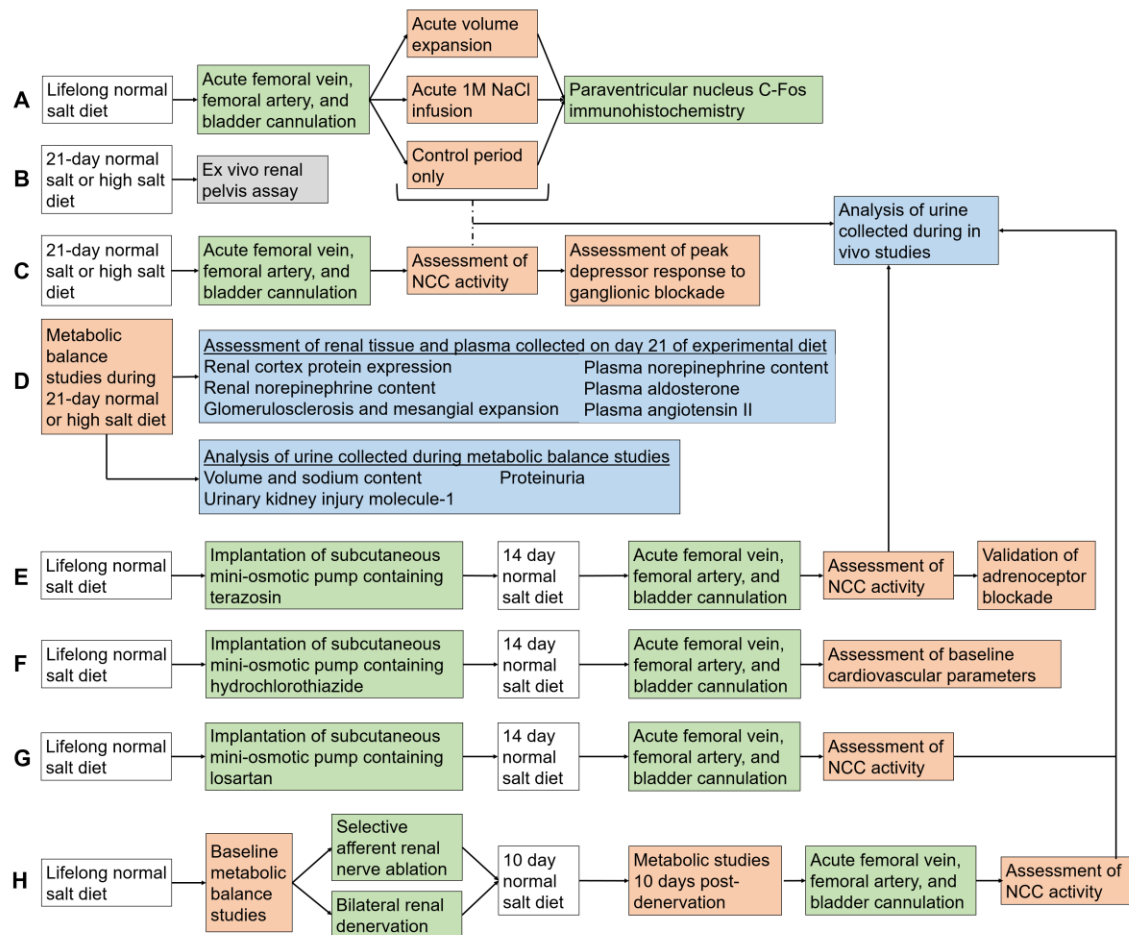


Figure 5.1. Overview of methods used in Chapter 5. All studies were performed using male Sprague Dawley rats aged (A-D) 3, 8 and 16 months old or (E-G) 3 and 16 months old only. White = dietary sodium assignments; green = surgical procedures; orange = in vivo studies; grey = ex vivo studies; blue = analytical techniques. $n = 6/\text{group}$ unless otherwise specified. Detailed protocols are described in Chapter 2: General Methods.

Results

Aged rats exhibit impaired cardiovascular, renal, and parvocellular PVN neuronal responses to an acute volume expansion

Male Sprague Dawley rats maintained on a normal salt diet exhibit a progressive age-related increase in blood pressure that was accompanied by a

decrease in heart rate in 16-month old rats (Figure 5.2A&B). An acute volume expansion evoked robust natriuretic and diuretic responses that allowed the excretion of almost 80% of the sodium and 95% of the fluid delivered in 3-month old rats (Figure 5.2C&D). In contrast, the natriuretic and diuretic responses to acute volume expansion were progressively blunted in 8-month old and 16-month old rats (Figure 5.2C&D). Despite the age-related impairment in renal excretory responses, an acute volume expansion did not alter blood pressure or heart rate in any age group (Figure 5.2A&B).

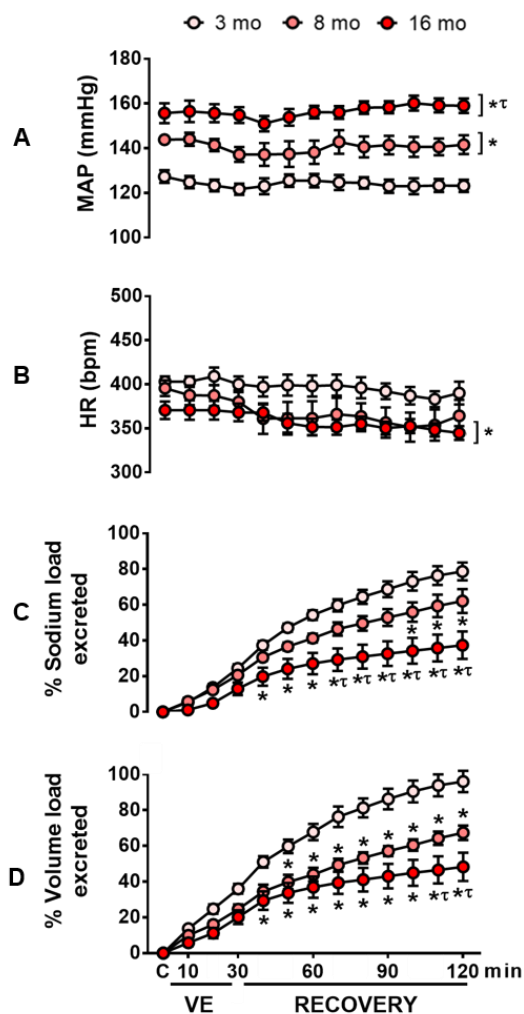


Figure 5.2. Impact of aging on the cardiovascular and renal responses to an acute volume expansion. (A) Mean arterial pressure (MAP; mmHg), (B) heart rate (HR; beats per minute [bpm]), (C) percentage of sodium load excreted and (D) percentage of volume load excreted during a control period (denoted 'C'), a 30-minute 5% body weight isotonic saline volume expansion (VE), and a 90-minute recovery period in conscious 3, 8, and 16-month old male Sprague Dawley (SD) rats maintained on a lifetime normal salt diet. In vivo data in this figure corresponds to Fos expression data in Figure 5.3 (VE groups only). $n = 6/\text{group}$. $*P < 0.05$ vs. respective 3-month group value; $\dagger P < 0.05$ vs. respective 8-month group value.

To assess the activation of sympathoinhibitory neurons in the PVN following surgical recovery (baseline) or acute volume expansion, the number of Fos-positive neurons was counted in parvocellular and magnocellular subnuclei. Baseline Fos staining in all parvocellular and magnocellular regions was minimal in 3-month old rats and was not altered in aged rats (Figure 5.3). An acute volume expansion evoked an increase in Fos staining in all paraventricular nucleus parvocellular and magnocellular subnuclei in 3-month old rats (Figure 5.3). While volume expansion-evoked Fos induction in magnocellular neurons was intact in 8-month and 16-month old rats, Fos staining in parvocellular subnuclei was significantly and progressively reduced following volume expansion in aged rats (Figure 5.3).

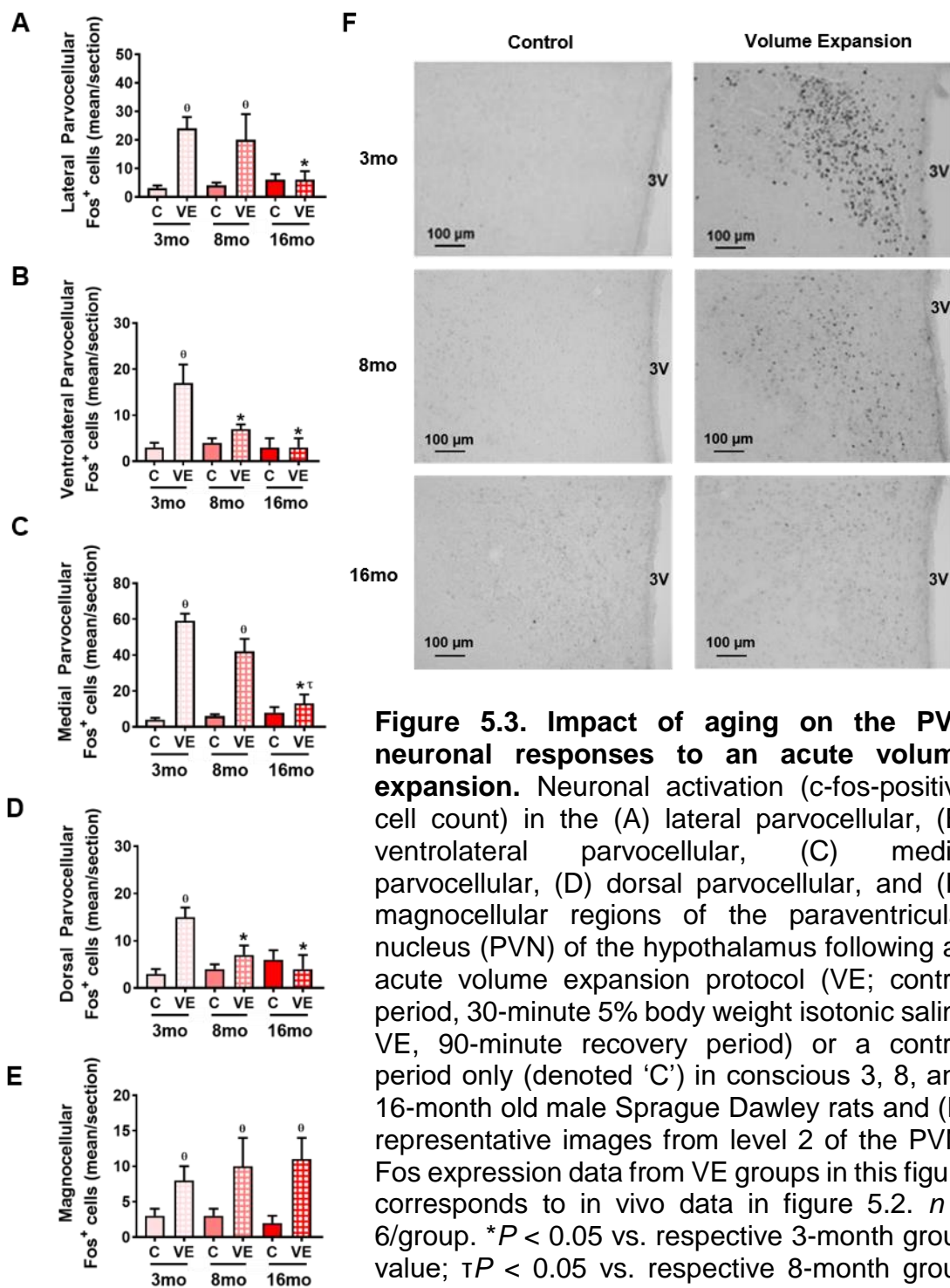


Figure 5.3. Impact of aging on the PVN neuronal responses to an acute volume expansion. Neuronal activation (c-fos-positive cell count) in the (A) lateral parvocellular, (B) ventrolateral parvocellular, (C) medial parvocellular, (D) dorsal parvocellular, and (E) magnocellular regions of the paraventricular nucleus (PVN) of the hypothalamus following an acute volume expansion protocol (VE; control period, 30-minute 5% body weight isotonic saline VE, 90-minute recovery period) or a control period only (denoted 'C') in conscious 3, 8, and 16-month old male Sprague Dawley rats and (F) representative images from level 2 of the PVN. Fos expression data from VE groups in this figure corresponds to in vivo data in figure 5.2. $n = 6/\text{group}$. $*P < 0.05$ vs. respective 3-month group value; $\tau P < 0.05$ vs. respective 8-month group value; $\theta P < 0.05$ vs. respective control group (denoted 'C').

Aged rats exhibit intact cardiovascular, renal, and parvocellular PVN neuronal responses to a hypertonic saline infusion

In male Sprague Dawley rats that underwent an acute 1M NaCl infusion, blood pressure increased and heart rate decreased to a similar degree in 8-month and 16-month old rats compared to 3-month old rats. An acute 1M NaCl infusion had no impact on blood pressure or heart rate in any age group (Figure 5.4A&B). In all age groups, a 1M NaCl infusion evoked profound natriuresis in the absence of diuresis (Figure 5.4C&D). A 1M NaCl infusion evoked an increase in Fos staining in all parvocellular and magnocellular regions of the PVN in 3-month old rats that was not altered in aged rats (Figure 5.5).

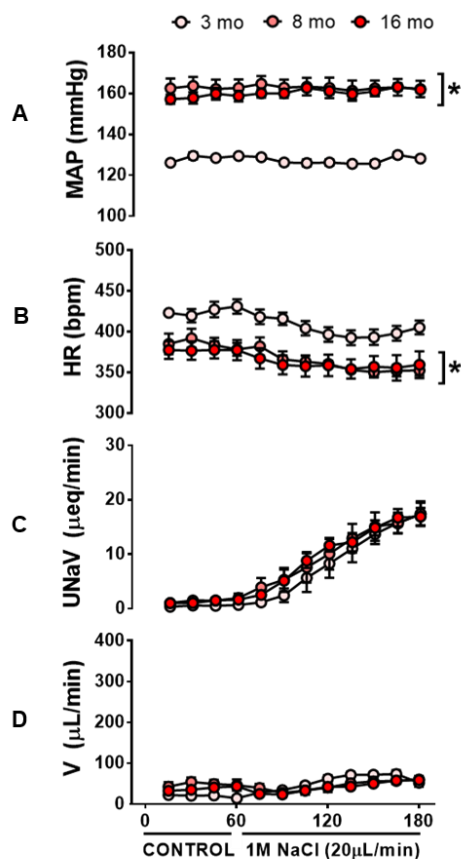


Figure 5.4. Impact of aging on the cardiovascular and renal responses to an acute 1M NaCl infusion. (A) Mean arterial pressure (MAP; mmHg), (B) heart rate (HR; beats per minute [bpm]), (C) natriuresis (UNaV; $\mu\text{eq}/\text{min}$) and (D) urinary flow rate (V; $\mu\text{L}/\text{min}$) excreted during a control period and a 2-hour 1M NaCl infusion ($20\mu\text{L}/\text{min}$) in conscious 3, 8, and 16-month old male Sprague Dawley rats maintained on a lifetime normal salt diet. In vivo data in this figure corresponds to Fos expression data in Figure 5.5 (1M groups only). $n = 6/\text{group}$. $*P < 0.05$ vs. respective 3-month group value.

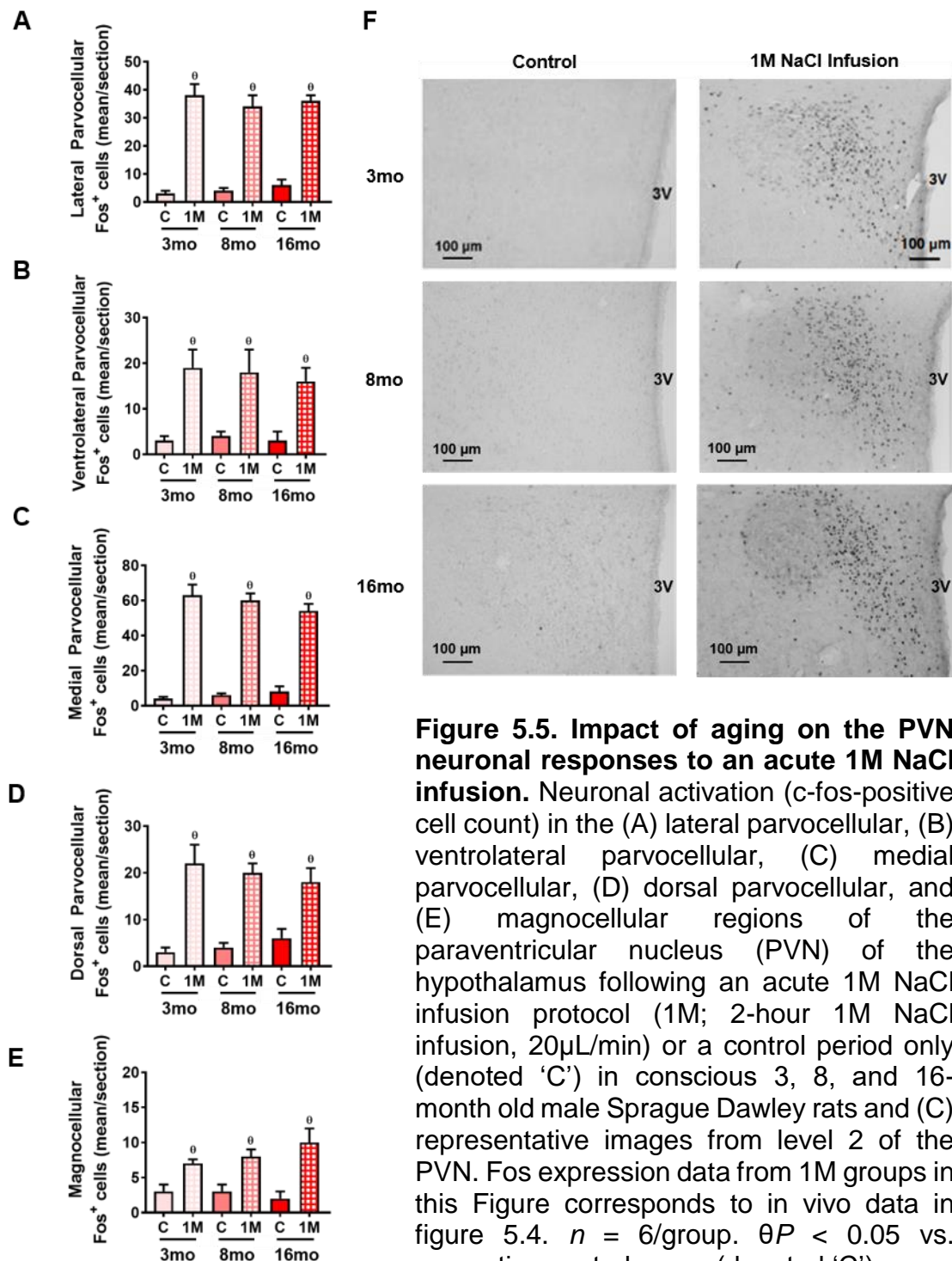


Figure 5.5. Impact of aging on the PVN neuronal responses to an acute 1M NaCl infusion. Neuronal activation (c-fos-positive cell count) in the (A) lateral parvocellular, (B) ventrolateral parvocellular, (C) medial parvocellular, (D) dorsal parvocellular, and (E) magnocellular regions of the paraventricular nucleus (PVN) of the hypothalamus following an acute 1M NaCl infusion protocol (1M; 2-hour 1M NaCl infusion, 20μL/min) or a control period only (denoted 'C') in conscious 3, 8, and 16-month old male Sprague Dawley rats and (F) representative images from level 2 of the PVN. Fos expression data from 1M groups in this Figure corresponds to in vivo data in figure 5.4. $n = 6/\text{group}$. $\theta P < 0.05$ vs. respective control group (denoted 'C').

Aged rats exhibit reduced ex vivo afferent renal nerve responsiveness and fail to enhance afferent renal nerve responsiveness during high salt intake

Norepinephrine, a general afferent renal nerve stimulus, evoked an increase in ex vivo afferent renal nerve release of substance P in 3-month old male Sprague Dawley rats that was blunted in 8-month and 16-month old rats (Figure 5.6A). Norepinephrine-evoked substance P release was enhanced during high salt intake in 3-month old rats, while this response was absent in 8-month and 16-month old rats (Figure 5.6A). In contrast, afferent renal nerve responsiveness to 450mM NaCl, a chemoreceptor stimulus, was not altered by age or diet (Figure 5.6B).

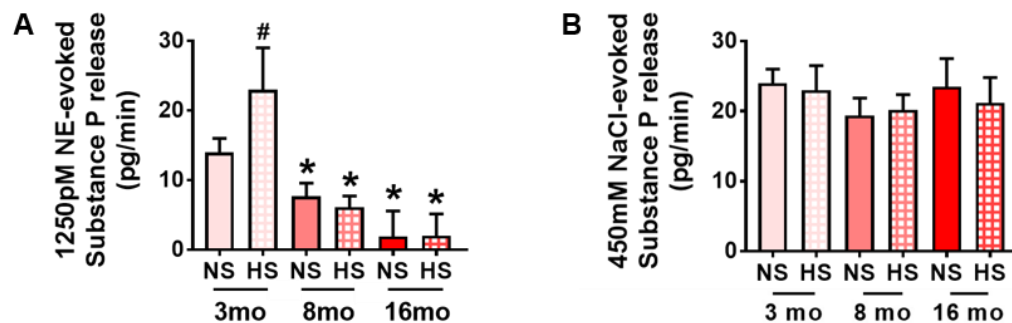


Figure 5.6. Impact of aging on ex vivo afferent renal nerve responsiveness. Ex vivo renal pelvis substance P release (pg/min) in response to (A) 1250 pM norepinephrine (NE) or (B) 450mM NaCl on day 21 of a normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet in male Sprague Dawley (SD) aged 3, 8, and 16 months (mo). $n = 6/\text{group}$. # $P < 0.05$ vs age-matched NS group value, * $P < 0.05$ vs. respective 3mo group value.

*Aged rats develop hypertension accompanied by increased sympathetic tone
and NCC activity during normal salt intake*

Blood pressure increased progressively with age in male Sprague Dawley rats maintained on a normal salt diet (Figure 5.7A). The age-related increases in blood pressure were accompanied by increases in global, vascular, and renal sympathetic tone, assessed as plasma norepinephrine concentration, peak depressor response to intravenous hexamethonium, and renal norepinephrine content, respectively (Figure 5.7B-D). Only 16-month old rats exhibited an increase in global sympathetic tone, while both 8-month old and 16-month old rats exhibited similar increases in vascular sympathetic tone compared to 3-month old rats (Figure 5.7B&C). In comparison, renal norepinephrine was increased in 8-month old rats and further elevated in 16-month old rats, demonstrating a progressive age-related increase in renal sympathetic tone (Figure 5.7D).

In vivo NCC activity, assessed as the peak natriuretic response to intravenous hydrochlorothiazide, is elevated to a similar degree in 8-month old and 16-month old male Sprague Dawley rats compared to 3-month old rats maintained on a normal salt diet (Figure 5.8A). Suggesting a differential age-related contribution of the NCC to blood pressure regulation, chronic antagonism of the NCC via subcutaneous hydrochlorothiazide infusion reduced blood pressure in 16-month-old rats, but not 3-month old rats (Figure 5.8B).

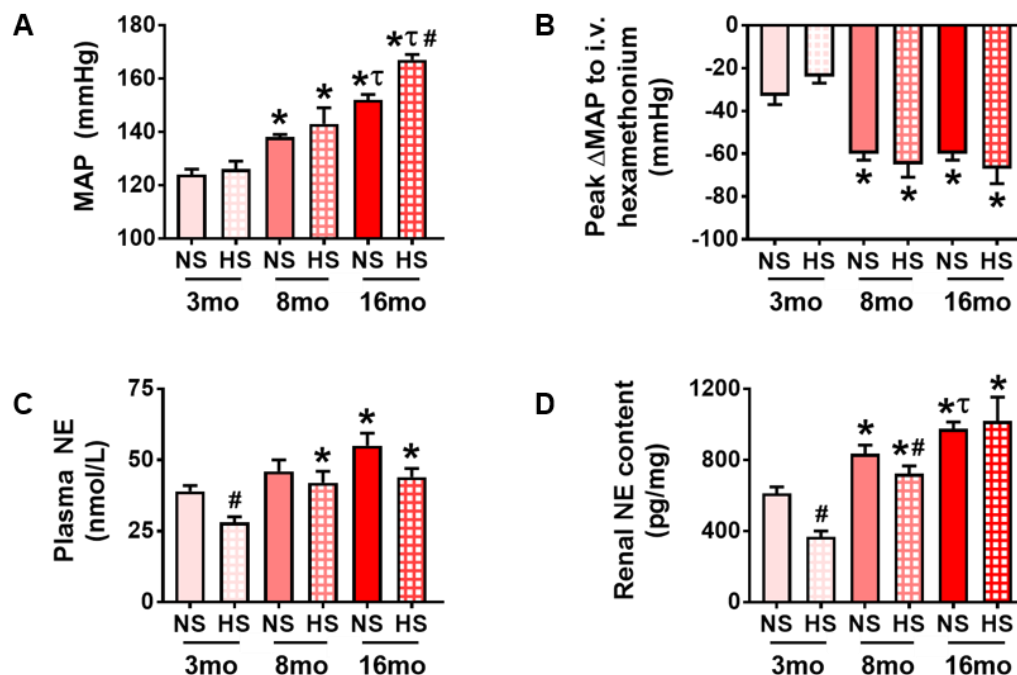


Figure 5.7. Impact of aging on blood pressure and sympathetic tone during normal and high salt intake. (A) Mean arterial pressure (MAP; mmHg) and (B) peak depressor response (Δ MAP, mmHg) to hexamethonium (30mg/kg, intravenous) assessed in conscious male Sprague Dawley rats aged 3, 8, and 16 months (mo) after a 21-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet and (C) plasma norepinephrine (NE) concentration (nmol/L) and (D) renal NE content (pg/mg) assessed following a 21-day NS or HS diet in separate groups of naïve male Sprague Dawley rats aged 3, 8, and 16mo. $n = 6$ /group. * $P < 0.05$ vs respective 3mo group, $\tau P < 0.05$ vs respective 8mo group, # $P < 0.05$ vs respective NS group.

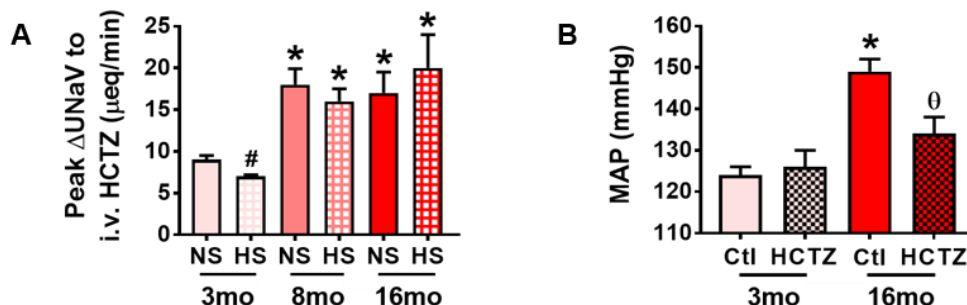


Figure 5.8. Impact of aging on NCC activity in vivo. (A) Peak natriuretic response (Δ UNaV, μ eq/min) to hydrochlorothiazide (HCTZ; 2mg/kg bolus followed by 1-hour 2mg/kg infusion, intravenous) in conscious male Sprague Dawley rats aged 3, 8, and 16 months (mo) following a 21-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet and (B) mean arterial pressure (MAP, mmHg) in conscious 3mo and 16mo male Sprague Dawley rats under control (Ctl) conditions or following a 14-day subcutaneous hydrochlorothiazide infusion (HCTZ; 4mg/kg/day). $n = 6$ /group except $n = 4$ for 16mo HCTZ infusion group. * $P < 0.05$ vs. respective 3mo group, # $P < 0.05$ vs. respective NS group, $\theta P < 0.05$ vs. respective control group.

To explore the signaling pathways underlying the observed physiological increase in NCC activity in aged animals, we assessed protein expression of the NCC and its regulatory kinases in the renal cortex of rats maintained on a normal salt diet, with a primary focus on the α_1 -adrenoceptor-gated WNK1/OxSR1 pathway described in Chapter 3. While total expression of the NCC, SPAK, and OxSR1 was not altered in 8-month and 16-month old rats and phosphorylated NCC (Thr53) decreased in 16-month old rats, total WNK1 expression and phosphorylated SPAK/OxSR1 increased with age (Figure 5.9). Further, in a small subset of 16-month old animals, chronic α_1 -antagonism reduced in vivo NCC activity and attenuated age-related hypertension (Figure 5.10).

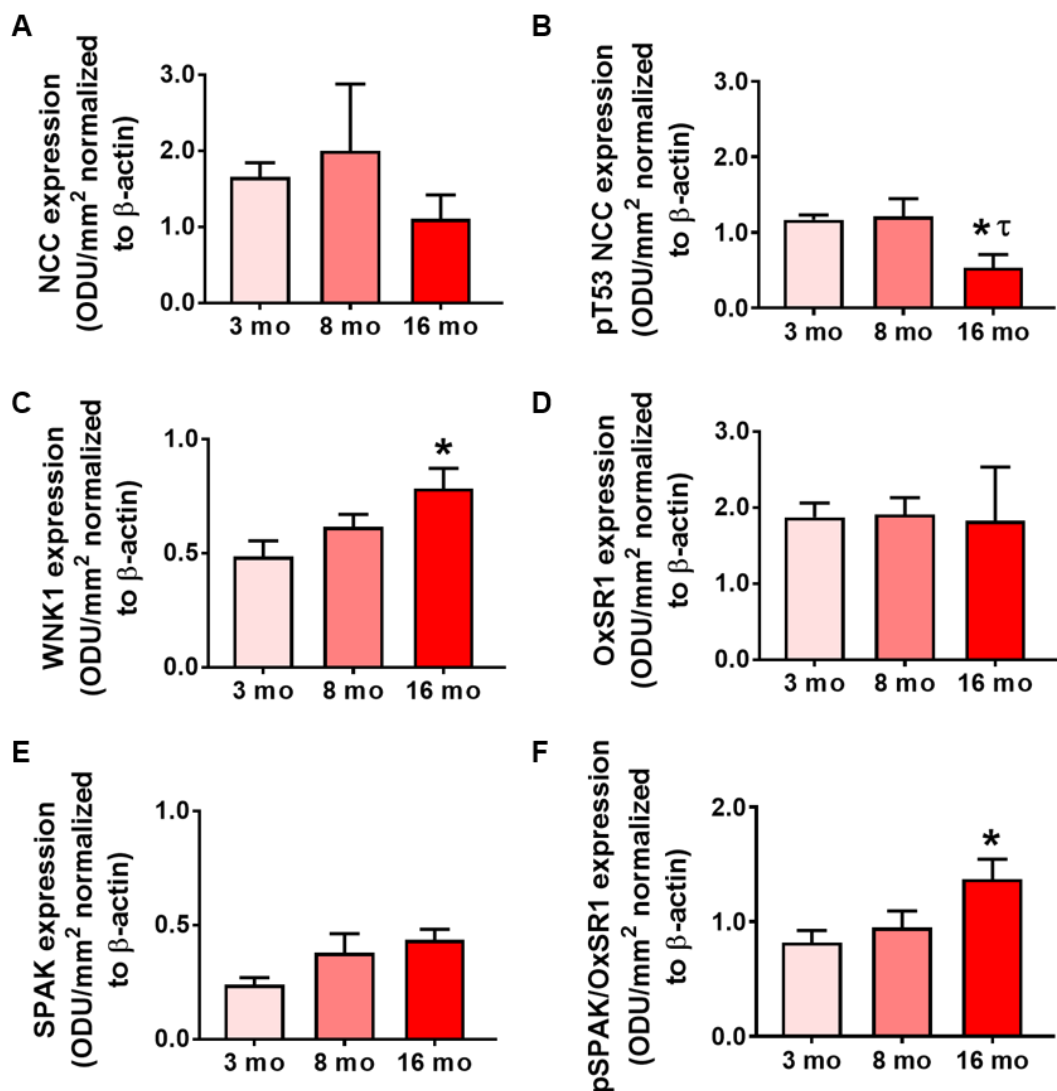
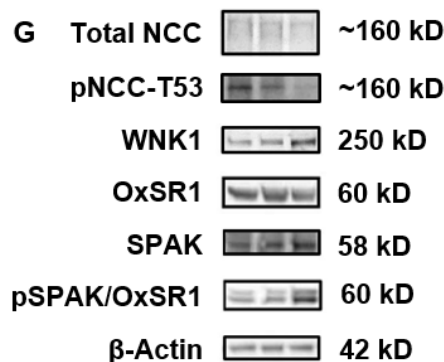


Figure 5.9. Impact of aging on the NCC regulatory kinase network. Quantification of renal cortex protein expression of (A) total NCC, (B) phosphorylated NCC (pT53), (C) total WNK1, (D) total OxSR1, (E) total SPAK, and (F) phosphorylated SPAK/OxSR1 normalized to β-actin and (G) representative immunoblots from 3, 8, and 16-month old (mo) male Sprague Dawley rats on a normal salt diet. $n = 4/\text{group}$. * $P < 0.05$ vs. respective 3-month group value; $\tau P < 0.05$ vs. respective 8-month group value.



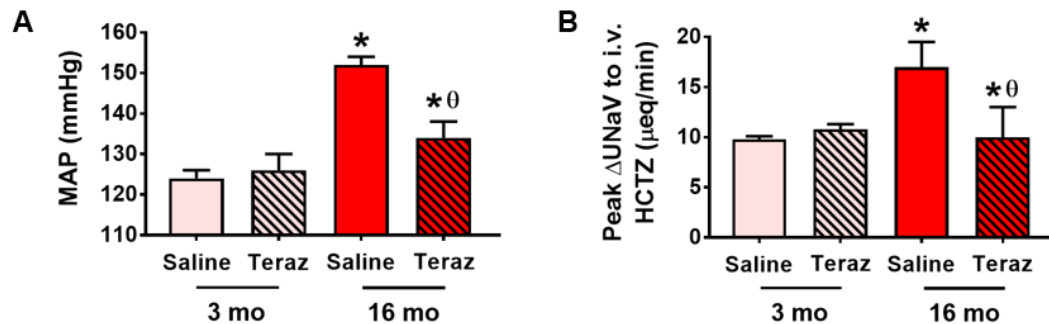


Figure 5.10. Impact of α_1 -adrenoceptor antagonism on age-related hypertension and NCC activity. (A) Mean arterial pressure (MAP; mmHg) and (B) peak natriuretic response (ΔUNaV) to intravenous hydrochlorothiazide (HCTZ; 2mg/kg bolus, 2mg/kg hour infusion) in conscious 3 and 16 month old male Sprague Dawley rats under control (ctl) conditions ($n = 6$) or following a 10-day subcutaneous infusion of terazosin (10mg/kg/day) ($n = 3$). * $P < 0.05$ vs. respective 3 month group, $\theta P < 0.05$ vs. respective control group.

Aged rats develop salt sensitivity of blood pressure accompanied by suppression of sympathetic tone and NCC activity during high salt intake

A 21-day high salt diet altered blood pressure, sympathetic tone, and NCC activity in an age-dependent manner. In normotensive 3-month old and hypertensive 8-month old male Sprague Dawley rats, high salt intake had no impact on blood pressure (Figure 5.7A). In contrast, age-related hypertension was exacerbated by a high salt diet in 16-month old rats, indicating the development of salt-sensitive hypertension (Figure 5.7A).

In 3-month old male Sprague Dawley rats, salt resistance was accompanied by dietary sodium-evoked suppression of global and renal sympathetic tone, but not vascular sympathetic tone (Figure 5.7C&D). However, while 8-month old rats also exhibited salt resistance, suppression of global and renal sympathetic tone were attenuated (Figure 5.7C&D). Dietary sodium-evoked suppression of global

sympathetic tone was impaired in 16-month old rats to a similar degree observed in 8-month old rats, while suppression of renal sympathetic tone was abolished only in the salt-sensitive 16-month old group (Figure 5.7C&D). Vascular sympathetic tone was not altered during increased dietary sodium intake at any age (Figure 5.7B).

High salt intake evoked the suppression of NCC activity in normotensive, salt-resistant 3-month old rats (Figure 5.8A). In contrast, hypertensive 8-month old rats and hypertensive, salt-sensitive 16-month old rats failed to suppress NCC activity during a high salt diet (Figure 5.8A).

Age-related hypertension and salt sensitivity develop independently of functional renal damage and alterations in the renin-angiotensin-aldosterone system

To determine the potential contribution of renal damage to age-related hypertension and salt sensitivity, we assessed kidney function and pathology by several measures in male Sprague Dawley rats. In a small subset of 3-month old, 8-month old, and 16-month old rats maintained on a normal salt diet, glomerular filtration rate was unchanged (Figure 5.11A&B). All age groups exhibited similar levels of proteinuria during normal salt intake, and a high salt diet increased proteinuria to a similar degree in all age groups (Figure 5.11C). Urinary kidney injury molecule-1, a marker of proximal tubular injury, and the glomerulosclerosis index and mesangial expansion score, two pathological changes that occur with

age and correlate with functional renal damage, were not altered by age or diet (Figure 5.11D&5.12).

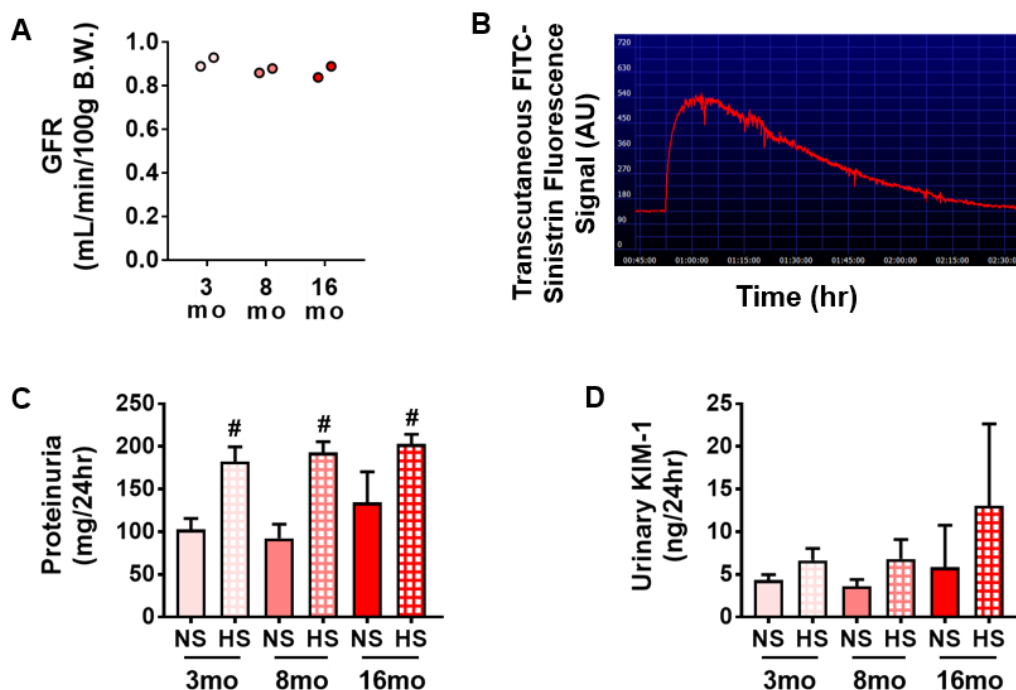


Figure 5.11. Impact of aging on renal function. (A) Glomerular filtration rate (GFR; mL/min/100g body weight [B.W.]) assessed using FITC-sinistrin clearance in a small subset ($n = 2/\text{group}$) of 3, 8, and 16 month (mo) male Sprague Dawley rats, (B) a representative FITC-sinistrin clearance curve, and (C) proteinuria (mg/24hr) and (D) urinary kidney injury molecule-1 (KIM-1; ng/24hr) assessed in urine collected from 3, 8, and 16mo male Sprague Dawley rats following a 21-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet ($n = 6/\text{group}$). # $P < 0.05$ vs respective NS group.

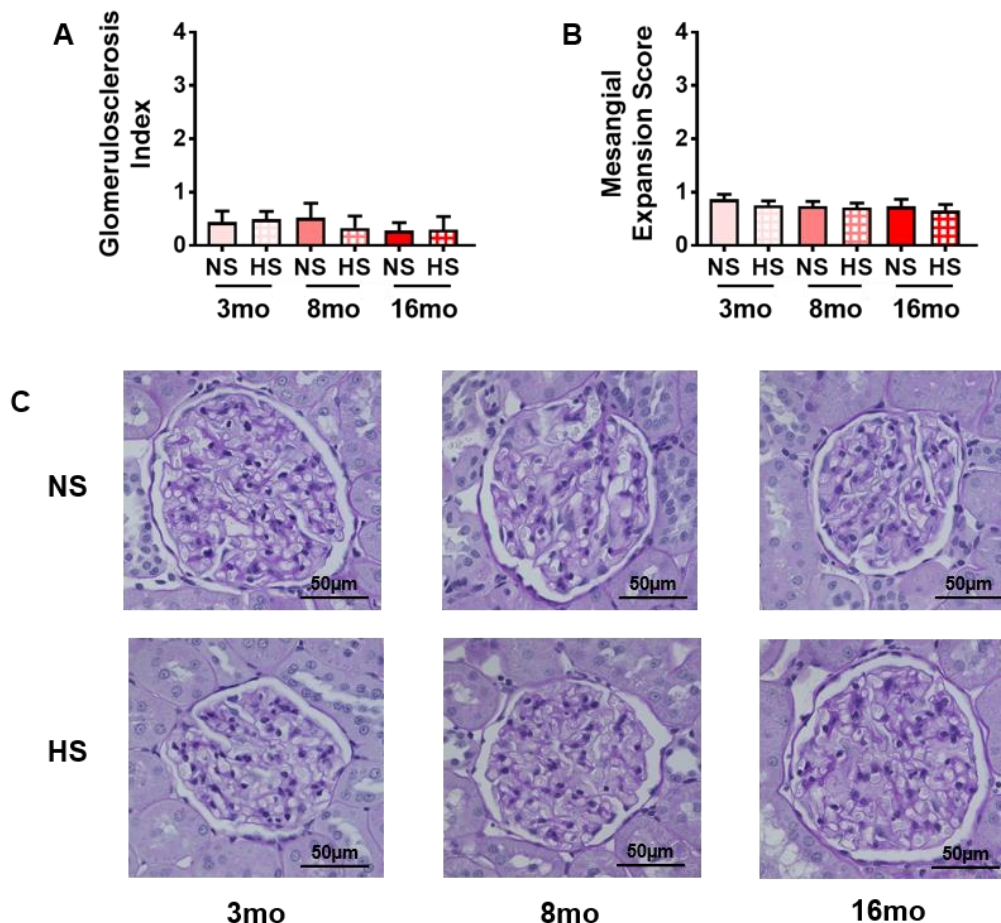


Figure 5.12. Impact of aging on renal histopathology. (A) Glomerulosclerosis index and (B) mesangial expansion score based on semiquantitative assessment of both measures in period acid-Schiff stained renal tissue obtained from 3, 8, and 16 month (mo) male Sprague Dawley rats following a 21-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet and (C) representative images from each group. $n = 6/\text{group}$; 42 glomeruli per rat assessed for both glomerulosclerosis and mesangial expansion.

Suggesting that the renin-angiotensin-aldosterone system is not involved in the development of age-related hypertension in male Sprague Dawley rats, plasma aldosterone and angiotensin II levels were similar in 3-month, 8-month, and 16-month old rats maintained on a normal salt diet (Figure 5.13A&B). Further, dietary sodium-evoked suppression of aldosterone was observed in 3-month old rats and

remained intact in 8-month old and 16-month old rats (Figure 5.13A). To investigate the role of the renin-angiotensin-aldosterone system in NCC regulation in aging animals, a small subset of 16-month old rats received a chronic subcutaneous infusion of losartan, an angiotensin II receptor blocker, prior to the assessment of NCC activity. Losartan infusion attenuated age-related hypertension but had no impact on NCC activity (Figure 5.13C&D).

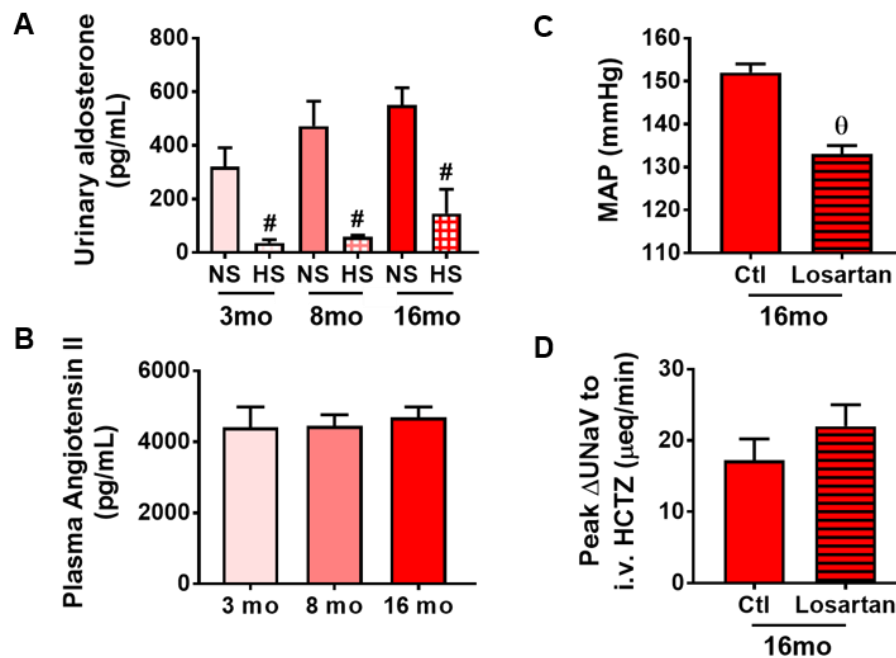


Figure 5.13. Role of the renin angiotensin aldosterone system in the regulation of blood pressure and NCC activity in aging rats. (A) Plasma aldosterone concentration (pg/mL) in 3, 8 and 16 month (mo) male Sprague Dawley (SD) rats maintained on a 21-day normal salt (NS; 0.6% NaCl) or high salt (HS; 4% NaCl) diet, (B) plasma angiotensin II concentration (pg/mL) in 3, 8, and 16mo male Sprague Dawley rats maintained on NS intake ($n = 6$ /group) and (C) mean arterial pressure (MAP; mmHg) and (D) peak natriuretic response (Δ UNaV, μ eq/min) to hydrochlorothiazide (HCTZ; 2mg/kg bolus followed by 1-hour 2mg/kg, intravenous) in conscious 16mo male SD rats under control (Ctl) conditions ($n = 6$) or following a 14-day subcutaneous infusion of losartan (3mg/kg/day) ($n = 3$). $\#P < 0.05$ vs. respective NS group, $\theta P < 0.05$ vs. control group.

*Bilateral renal denervation, but not selective afferent renal nerve ablation,
attenuates age-related hypertension*

Bilateral renal denervation and selective afferent renal nerve ablation were used to dissect the roles of the renal sympathetic nerves and afferent renal nerves on sodium balance, NCC activity, and blood pressure regulation in 3-month old and 16-month old male Sprague Dawley rats during normal salt intake. Selective afferent renal nerve ablation had no impact on blood pressure, sodium balance, or NCC activity in either age group (Figure 5.14A-C). In contrast, bilateral renal denervation improved sodium balance and attenuated age-related hypertension in 16-month old rats but had no impact on cardiovascular or renal excretory function in 3-month old rats (Figure 5.14A&B). Somewhat unexpectedly, bilateral renal denervation and selective afferent renal nerve ablation had no impact on in vivo NCC activity in 3-month old rats or 16-month old rats (Figure 5.14C).

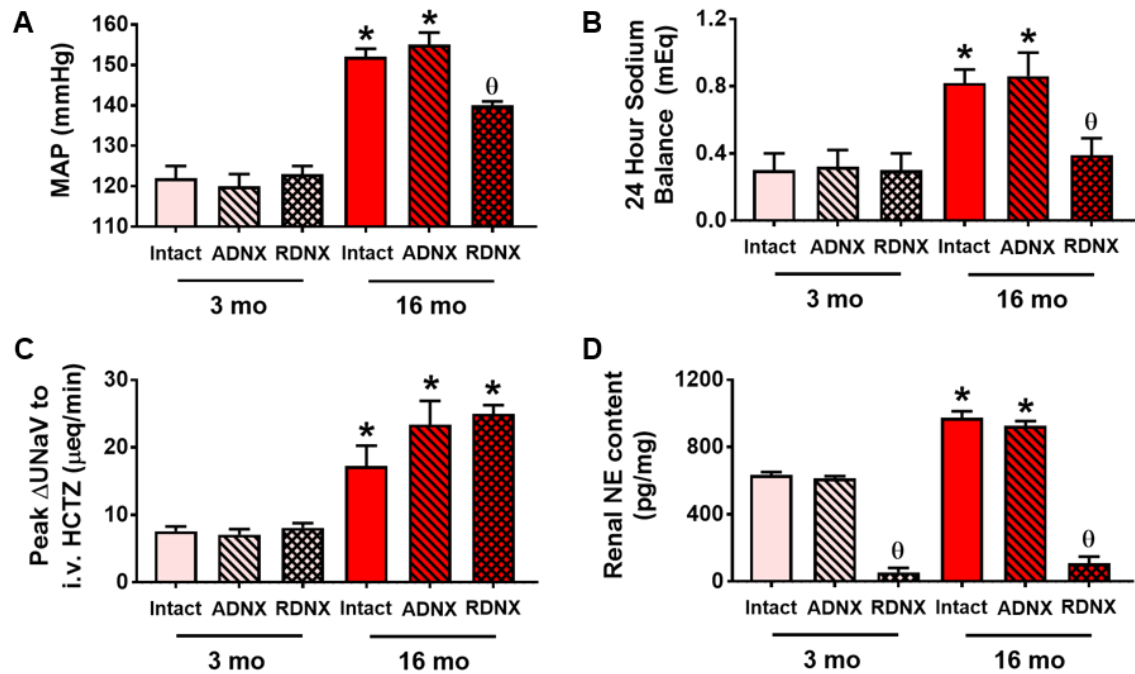


Figure 5.14. Role of the renal nerves in the regulation of blood pressure, sodium balance, and NCC activity in aging rats. (A) Mean arterial pressure (MAP; mmHg), (B) 24 hour sodium balance (mEq), and (C) peak natriuretic response (Δ UNaV, μ eq/min) to hydrochlorothiazide (HCTZ; 2mg/kg bolus followed by 1-hour 2mg/kg, intravenous) in conscious 3 and 16 month old (mo) male Sprague Dawley rats with intact renal nerves or 10 days after selective afferent renal denervation (ADNX) or bilateral renal denervation (RDNX) and (D) renal norepinephrine content assessed in renal tissue collected immediately after the assessment of MAP and NCC activity. $n = 6$ /group. * $P < 0.05$ vs respective NS group, $\theta P < 0.05$ vs respective intact group.

Discussion

The current studies were designed to investigate whether changes in the integrated renal and neural mechanisms that modulate sodium homeostasis and blood pressure contribute to the development of age-related hypertension and salt sensitivity of blood pressure. Previous studies have demonstrated that norepinephrine-evoked NCC activity promotes salt-sensitive hypertension, and others have delineated an acute afferent renal nerve-mediated sympathoinhibitory reno-renal reflex that suppresses renal sympathetic outflow and promotes natriuresis and normotension (Kopp, 1993; Johns & Abdulla, 2013; Johns, 2014; Kopp, 2015). Further, a protective role for sensory afferent signaling in salt resistance has been suggested using non-selective global sensory denervation techniques (Wang et al., 1998; Wang et al., 2001; Kopp et al., 2003). In Chapter 3, we established that norepinephrine promotes NCC activity via an α_1 -adrenoceptor-gated WNK1-OxSR1 signaling pathway, ultimately driving the development of salt-sensitive hypertension in 3-month old rats. In Chapter 4, we demonstrated in 3-month old rats that the mechanosensitive afferent renal nerves are required for appropriate sympathoinhibitory reno-renal reflex activity, perhaps via activation of parvocellular neurons in the PVN, and provided evidence that the afferent renal nerves are a specific and critical component of salt resistance that is impaired in salt-sensitive animal models. The current studies extend these observations into a model of normal aging and demonstrate that aged rats develop salt-sensitive hypertension driven in part by pathological changes that include 1) a

selective impairment in the mechanosensitive sympathoinhibitory reno-renal reflex, 2) a reduction in afferent renal nerve activity and a failure to increase afferent renal nerve activity during high salt intake, which again likely reflects a selective impairment in the mechanosensitive afferent renal nerves, and 3) an increase in sympathetic outflow and NCC activity, likely via an α_1 -adrenoceptor gated WNK1-OxSR1 signaling pathway, and a failure to suppress sympathetic tone and NCC activity in response to a high salt diet.

Age-related hypertension and salt sensitivity are difficult to model in rodents. Hypertension and salt sensitivity are typically investigated using young animal models in which genetic or experimental manipulations drive pathophysiology (Leong et al., 2015). Models of aging are used infrequently in the study of hypertension and salt sensitivity, perhaps in part because these models are either hypertension-resistant or develop hypertension in the presence of potentially confounding pathological changes to the systems involved in blood pressure regulation, including the kidney (Coleman et al., 1977; Bilusic et al., 2008). In these studies, we used Sprague Dawley rats as a model of normal aging. Multiple studies have demonstrated that young adult (3-month) Sprague Dawley rats are salt-resistant (Kapusta et al., 2012; Kapusta et al., 2013; Wainford et al., 2015) but develop salt sensitivity with norepinephrine infusion (Walsh et al., 2016). These findings may reflect similarities with human salt sensitivity of blood pressure, which is less common in younger individuals but becomes increasingly common alongside elevated sympathetic tone and hypertension in aged individuals (Luft et

al., 1987; Luft et al., 1991; Esler et al., 1995; Esler et al., 2002; Writing Group et al., 2016; Whelton et al., 2018). Further, while aging Sprague Dawley rats develop renal damage associated with structural alterations, such as glomerulosclerosis, and functional alterations, including reduced glomerular filtration rate, these features are primarily observed after the age of 20 months (Owen & Heywood, 1986; Erdely et al., 2003). By using 3-month, 8-month, and 16-month old rats, we were able to temporally isolate the development of hypertension and salt sensitivity of blood pressure from the potentially confounding effect of functional renal damage, which could alter renal sodium handling and renal nerve function.

To investigate the impact of aging on the acute reno-renal reflexes, we first used the classical sympathoinhibitory challenge of an acute volume expansion (Schad & Seller, 1976; Ricksten & Thoren, 1980; Haselton et al., 1994; Kapusta et al., 2012). We observed a progressive increase in baseline blood pressure in 8-month and 16-month old rats, indicating the development of age-related hypertension. Further, an acute volume expansion produced a robust natriuretic and diuretic response in 3-month old animals that was significantly and progressively blunted in 8-month and 16-month old animals. While the current studies were conducted primarily in male rats, similar impairments were observed in preliminary studies in 8-month old female rats. In Chapter 4, we observed that an acute volume expansion evokes an increase in renal pelvic pressure in anesthetized 3-month old male Sprague Dawley rats and that the afferent renal nerves are required for maximal volume expansion-evoked natriuresis and diuresis

(Figure 4.2). These findings, combined with prior work demonstrating that an acute volume expansion activates afferent renal nerve mechanoreceptors that selectively respond to increased renal pelvic pressure (Chien et al., 2000), strongly suggest that the age-related reduction in volume expansion-evoked natriuresis and diuresis reflects an impairment in the mechanosensitive afferent renal nerves.

Consistent with this interpretation, the robust increase in Fos-positive cell bodies observed in all parvocellular PVN regions following volume expansion in 3-month old rats, which requires intact afferent renal nerves (Chapter 4, Figure 4.2), was progressively attenuated in 8-month and 16-month old rats. Although volume expansion evoked an increase in magnocellular PVN Fos staining in 3-month old rats, this response remained intact in aged rats, suggesting that age-related impairments in volume expansion-evoked natriuresis and diuresis do not reflect altered magnocellular neuronal activation. Previous studies have established that sympathoinhibitory parvocellular neurons in the PVN are activated by an acute volume expansion and in turn promote the suppression of sympathetic outflow and a robust natriuretic response (Haselton et al., 1994; Randolph et al., 1998; Ng et al., 2004). While the functional nature of the parvocellular PVN neurons activated following volume expansion in aged animals in the current studies requires further clarification, the associated impairment in volume expansion-evoked natriuresis and diuresis suggests that the age-related reduction in parvocellular Fos staining in the PVN could reflect impaired activation of sympathoinhibitory neurons. Supporting this interpretation, a previous electrophysiological study demonstrated

that the suppression of renal sympathetic nerve activity following an acute dextran volume expansion was blunted in aged dogs (Hajduczuk et al., 1991a). As indicated in Chapter 4, further studies in each age group will be required to confirm that the observed Fos staining reflects functional activation of sympathoinhibitory parvocellular PVN neurons.

Collectively, our observations suggest that aging is associated with a selective impairment in the mechanosensitive afferent renal nerves, resulting in blunted natriuretic and diuretic responses to an acute volume expansion. Further, these impairments are associated with reduced Fos staining in the PVN, lending support to the putative role of the PVN in the central integration of the sympathoinhibitory reno-renal reflex proposed in Chapter 4. While our findings, particularly in light of the studies described in Chapter 4, suggest a specific impairment in sensory signaling originating in the kidney, an acute volume expansion also stimulates non-renal sensory afferent signaling pathways that activate parvocellular neurons in the PVN and suppress renal sympathetic outflow, including those activated by atrial stretch (Karim et al., 1972; Pyner et al., 2002). The impact of aging on these non-renal sensory afferent pathways has not been established and could contribute pathologically to the impaired homeostatic responses to volume expansion if impaired, or protectively to the residual natriuretic and diuretic responses to volume expansion observed in aged rats if intact.

To assess the function of the chemosensitive afferent renal nerves, we used a 1M NaCl infusion that does not increase renal pelvic pressure in 3-month old anesthetized Sprague Dawley rats (Chapter 4, Figure 4.6). The chemosensitive afferent renal nerves respond to changes in the chemical composition of fluid in the renal pelvis, including alterations in sodium concentration (Recordati et al., 1980; Recordati et al., 1981). Given that the fluid that reaches the renal pelvis is not further altered before excretion as urine, our observation that a 1M NaCl infusion yields a highly concentrated urine output via a robust natriuresis in the absence of diuresis suggests that the 1M NaCl infusion should preferentially activate chemoreceptors present in the renal pelvis. Age had no impact on the profound natriuresis evoked by a 1M NaCl infusion, and rats in all age groups exhibited indistinguishable increases in Fos-positive cell count in all parvocellular and magnocellular regions of the PVN, suggesting that the chemosensitive afferent renal nerves remain functional. Of note, the chemosensitive afferent renal nerves have been implicated in a sympathoexcitatory reno-renal reflex that promotes sodium retention and has primarily been observed in disease states including renal ischemia and heart failure (Recordati et al., 1982; Rogenes, 1982; Zheng et al., 2018). While 16-month old rats appear to exhibit normal natriuretic responses to a 1M NaCl infusion, investigation of these responses in older animals (>20 months) could yield important information, as renal damage becomes more common and more severe and could potentially promote sympathoexcitatory reno-renal reflex activity (Owen & Heywood, 1986; Erdely et al., 2003).

While the functional nature of the parvocellular PVN neurons activated by a 1M NaCl infusion has not been characterized, a previous study demonstrating that 1M NaCl infusion suppresses central sympathetic outflow indicates that some of the Fos-positive parvocellular neurons could be sympathoinhibitory in nature (Wainford et al., 2013). Importantly, our previous observations that 1M NaCl-evoked parvocellular neuron activation in the PVN and natriuresis remain intact following selective ablation of the afferent renal nerves (Chapter 4, Figure 4.5) and bilateral renal denervation (Wainford et al., 2013) suggest that non-renal sensory signaling pathways may mediate these responses. Although our interpretation of the 1M NaCl studies is limited somewhat by the unusually high blood pressure of the 8-month age group, the fully intact responses observed in the 16-month age group, in which blood pressure was comparable to similarly aged rats in all in vivo studies, and subsequent ex vivo studies of afferent renal nerve responsiveness suggest that chemosensitive afferent renal nerve function is not altered in aged rats.

To validate the potential age-related reduction in afferent renal nerve activity in isolation, an ex vivo renal pelvic preparation was used. While technical limitations prevent the experimental manipulation of renal pelvic pressure in this preparation, the afferent renal nerves were stimulated by treating the isolated renal pelvis with norepinephrine, a general stimulus that activates the afferent renal nerves via α_1 -adrenoceptors, and 450mM NaCl, a selective chemoreceptor stimulus near the upper limit of urinary sodium concentration (Kopp et al., 2007).

Afferent renal nerve responsiveness was assessed as the release of substance P, which plays a mechanistic role in afferent renal nerve activation (Kopp & Smith, 1993; Kopp et al., 1997; Kopp et al., 2000). In rats maintained on a normal salt diet, there was a reduction in norepinephrine-evoked substance P release in 8-month old and 16-month old rats, while sodium-evoked substance P release was not altered with age. This suggests that aging is associated with a reduction in baseline afferent renal nerve function that does not reflect an impairment in the chemosensitive afferent renal nerves, indirectly supporting our *in vivo* observation of a selective impairment in the mechanosensitive afferent renal nerve-mediated sympathoinhibitory reno-renal reflex.

Consistent with previous studies, 3-month old Sprague Dawley rats exhibited enhanced afferent renal nerve responsiveness to norepinephrine during high salt intake (Kopp et al., 2009). In contrast, afferent renal nerve responsiveness to sodium was not altered during high salt intake in 3-month old rats. We previously demonstrated that this selective enhancement is also present in Dahl Salt Resistant rats but abolished in Dahl Salt Sensitive rats, suggesting that a failure to enhance afferent renal nerve activity during high salt intake may be a pathological feature of salt-sensitive hypertension (Chapter 4, Figure 4.7). Here, we observed that hypertensive 8-month old rats and salt-sensitive, hypertensive 16-month old rats fail to increase afferent renal nerve responsiveness to norepinephrine, but not to the direct chemoreceptor stimulus. Together with our *in vivo* data, these findings suggest that young rats exhibit a selective

enhancement of the mechanosensitive afferent renal nerve-mediated reno-renal reflex that is impaired in aged animals.

To determine whether age-related impairments in acute afferent renal nerve activation were associated with alterations in the long-term regulation of blood pressure, sodium homeostasis, and sympathetic tone, we assessed these parameters in rats fed on a 21-day normal versus high salt diet. In rats maintained on a normal salt diet, age-related hypertension was associated with an increase in global, vascular, and renal sympathetic tone. While blood pressure and renal sympathetic tone increased progressively in each age group, global and vascular sympathetic tone did not increase further after 8 months of age. Interestingly, while human aging is associated with increased global sympathetic tone, an organ-specific study of norepinephrine turnover suggested that renal sympathetic outflow is not altered with age in humans (Esler et al., 1995; Esler et al., 2002). However, increased renal norepinephrine content is linked to sodium retention and hypertension in rodent models of hypertension, and baseline renal sympathetic nerve activity is elevated in aged beagles (Hajduczuk et al., 1991a; Hajduczuk et al., 1991b; Pinto et al., 2011). In humans, several studies have demonstrated that the responsiveness of the renal sympathetic nerves to various stimuli increases with age (Kuipers et al., 2009; Patel et al., 2013). A specific role for increased renal sympathetic tone in the development of age-related hypertension in Sprague Dawley rats is supported by our observation of a reduction in blood pressure, with a corresponding improvement in sodium balance, following removal of both the

efferent renal sympathetic nerves and afferent renal nerves via bilateral renal denervation in 16-month old rats. These findings are supported by a study demonstrating the efficacy of renal denervation in elderly hypertensive patients (Ziegler et al., 2015). Selective ablation of the afferent renal nerves had no impact on blood pressure or sodium homeostasis in 16-month old rats, which may in part reflect reduced basal afferent renal nerve activity in 16-month old rats. Importantly, this finding suggests that the beneficial effects of bilateral renal denervation, which removes both the efferent renal sympathetic nerves and the afferent renal nerves, are due to removal of the renal sympathetic nerves. Selective afferent renal nerve ablation and bilateral renal denervation had no impact on blood pressure in 3-month old rats, perhaps reflecting a role of the renal nerves in the responses to altered homeostasis rather than baseline blood pressure regulation during normal salt intake.

In 3-month old rats, which exhibited enhanced afferent renal nerve activity during high salt intake, a high salt diet resulted in suppression of global, vascular, and renal sympathetic tone and had no impact on blood pressure. These findings are consistent with previous studies establishing the role of dietary sodium-evoked sympathoinhibition in salt resistance in young Sprague Dawley rats (Walsh et al., 2016). In 8-month old rats, although dietary sodium-evoked suppression of global and renal sympathetic tone is attenuated and suppression of vascular sympathetic tone is abolished, a high salt diet did not exacerbate age-related hypertension. In contrast, 16-month old rats developed salt-sensitive hypertension and while these

animals exhibited impairments in dietary sodium-evoked suppression of global and vascular tone similar to those observed in 8-month old rats, suppression of renal sympathetic outflow was fully abolished only in 16-month old rats. Our observation that age-related hypertension and salt sensitivity are associated with baseline sympathoexcitation and impaired dietary sodium-evoked sympathoinhibition assessed in multiple sympathetic nervous system targets is consistent with our understanding of hypertension as a disease with complex, multifactorial etiology. However, along with our renal denervation studies, the progressive age-related increase in baseline renal sympathetic outflow and impairment in dietary sodium-evoked renal sympathoinhibition that accompany salt-sensitive hypertension in aged rats suggest that the renal sympathetic nerves play a pathophysiological role in age-related hypertension and salt sensitivity. Further, the development of impaired acute afferent renal nerve responsiveness in 8-month old rats prior to the development of salt sensitivity of blood pressure, combined with our previous observation that the afferent renal nerves are required for the maintenance of salt resistance in young Sprague Dawley rats (Chapter 4, Figure 4.8), support the proposed role for reduced afferent renal nerve function in the development of salt-sensitive hypertension in aged rats. Taken together, these findings support the hypothesis that age-related impairments in the mechanosensitive afferent renal nerve sympathoinhibitory reno-renal reflex may promote sympathoexcitation, sodium retention, and age-related hypertension.

The renal sympathetic nerves release norepinephrine, which can influence sodium homeostasis and blood pressure through its effects on renal sodium transporters and the renal vasculature. While norepinephrine acts at α_1 -adrenoceptors in the renal vasculature to promote vasoconstriction, resulting in reductions in renal plasma flow and glomerular filtration rate that enhance sodium reabsorption, our preliminary findings in small subgroups of aged animals indicate that glomerular filtration rate is not altered in 8-month old and 16-month old rats during normal salt intake. Although these findings must be interpreted cautiously due to a small sample size, previous studies have demonstrated that a functionally relevant age-related reduction in glomerular filtration rate in Sprague Dawley rats does not develop until 24 months of age (Lim et al., 2014), and suggests that age-related hypertension develops independently of changes in renal hemodynamics.

While the absence of an age-related alteration in glomerular filtration rate must be confirmed in a larger group of animals, several other findings also suggest that renal function is not impaired in our model of aging. Proteinuria, an indicator of renal damage, was similar in all age groups during normal salt intake and increased to a similar degree during high salt intake in all age groups. The similarity between age groups suggests that this measure of renal function is not linked to the age-related alterations in renal sodium handling and blood pressure regulation observed in the current studies. Urinary kidney injury molecule-1, a marker of proximal tubule injury, was not affected by age or dietary sodium intake was not altered during high salt intake. Glomerulosclerosis index and mesangial expansion

score, two measures of histopathological changes observed in aging humans that may contribute to altered renal function, were also similar in all age groups and were not altered by dietary sodium intake. Together, these data suggest that age-related sympathoexcitation, sodium retention, and hypertension develop independently of functional renal damage.

In the renal tubules, norepinephrine can promote the activity of several sodium transporters both directly and indirectly, by stimulating renin secretion and activating the renin angiotensin aldosterone system. While studies of human aging indicate that systemic renin angiotensin aldosterone system activity is reduced with age (Weidmann et al., 1975; Noth et al., 1977; Messerli et al., 1983), aging in several animal models is characterized by enhanced sensitivity of the intrarenal renin angiotensin aldosterone system (Tank et al., 1994; Thompson et al., 2000). In the current study, in which Sprague Dawley rats were selected as a model of normal aging and develop increases in sympathetic tone, blood pressure, and salt sensitivity that mimic those observed in human aging, plasma aldosterone and angiotensin II were not altered in 8-month or 16-month old rats, and dietary sodium-evoked aldosterone suppression remained intact. These data suggest that the renin angiotensin aldosterone system does not drive age-related hypertension and salt sensitivity in Sprague Dawley rats and support a specific role for increased sympathetic nervous system activity.

Previous studies have demonstrated that the direct influence of the sympathetic nervous system on NCC activity is particularly relevant in the context

of varying dietary sodium intake (Mu et al., 2011; Terker et al., 2014; Walsh et al., 2016). Infusion of norepinephrine increases NCC expression and phosphorylation in mice and prevents the suppression of NCC expression and activity in rats, driving the development of salt-sensitive hypertension independently of the renin angiotensin aldosterone system (Mu et al., 2011; Terker et al., 2014; Walsh et al., 2016). In the current studies, aging animals exhibiting a progressive increase in both blood pressure and renal norepinephrine content also exhibited increased NCC activity during normal salt intake. Further, while salt-resistant, normotensive 3-month old rats exhibit dietary sodium-evoked suppression of NCC activity, a finding that is consistent with previous studies, NCC activity is not altered by dietary sodium intake and remains elevated in 8-month and 16-month old animals that also fail to suppress renal norepinephrine content.

These findings add to a conflicted body of literature regarding age-related changes in NCC regulation. A study of aging Fischer Brown Norway rats suggested that aging is associated with an increase in NCC expression, and separate studies have established that these rats develop age-related hypertension (Tian et al., 2006; Chugh et al., 2012; Chugh et al., 2013; Pokkunuri et al., 2015). In contrast, the aged Sabra rat exhibits a decrease in distal convoluted tubule Na^+/K^+ -ATPase activity, which drives and typically correlates directly with NCC activity (Scherzer et al., 2015). It is worth noting that blood pressure was not measured in the study of Sabra rats and the authors did not report whether the hypertensive-resistant or hypertension-prone Sabra strain was

used (Ben-Ishay et al., 1980). This raises the possibility that the age-related decrease in Na^+/K^+ -ATPase activity, and likely NCC activity, plays a protective or compensatory role in the hypertension-resistant or hypertension-prone rat, respectively. This possibility is supported by another study in which aging C57Bl6/CBA/129 mice, which do not develop age-related hypertension, exhibited decreased baseline NCC activity (Tiwari et al., 2009). Importantly, aged animals exhibited enhanced increases in both NCC activity and blood pressure during the exogenous administration of angiotensin II (Tiwari et al., 2009), supporting our observation that aging is associated with impairments in the dynamic regulation of the NCC that are relevant to blood pressure regulation.

The signaling pathways through which norepinephrine influences NCC activity remain controversial, but in general NCC activation involves WNK kinases upstream of SPAK and OxSR1, which directly phosphorylate and activate the NCC. Initial studies of sympathetically-mediated NCC regulation implicated a β_2 -adrenoceptor gated pathway involving WNK4 and SPAK, but subsequent work failed to replicate the role of WNK4 and a third study described a synergistic role for α_1 - and β_2 -adrenoceptors in a signaling pathway that requires OxSR1, but not SPAK (Mu et al., 2011; Uchida et al., 2012; Terker et al., 2014). Our studies in young Sprague Dawley rats demonstrate that norepinephrine infusion evokes NCC activation mediated by an α_1 -adrenoceptor-gated WNK1-OxSR1 signaling pathway that drives the development of salt-sensitive hypertension (see Chapter 3). Although further studies are required to determine the specific signaling

pathways that mediate age-related increases in NCC activity, our findings at the protein level suggest that WNK1 and phosphorylated SPAK/OxSR1, both of which increase in aged rats during normal salt intake, could be involved. Unexpectedly, however, 16-month old animals exhibited decreased NCC phosphorylation at the Thr53 residue. While this observation could reflect a compensatory response to increased NCC activity and sodium retention, further studies are required to assess other factors that modulate NCC activity, including phosphorylation at different residues, ubiquitinylation, and alterations in sub-cellular localization of the NCC. While the observed reduction in blood pressure and NCC activity in a small group of 16-month old rats treated with an α_1 -antagonist suggests that α_1 -adrenoceptors play a role in the age-related increase in NCC activity, these findings must be replicated in a larger group and the selectivity of α_1 -adrenoceptor involvement must be tested. Importantly, chronic NCC antagonism reduces blood pressure in 16-month old rats but not 3-month old rats, suggesting that the NCC may play a specific role in blood pressure regulation in aged animals.

In light of the established role of norepinephrine-mediated NCC regulation in salt-sensitive hypertension and our data suggesting that age-related hypertension is characterized by increased NCC activity and renal sympathetic tone and attenuated by both NCC antagonism and renal denervation, our observation that NCC activity was not reduced following renal denervation in 16-month old rats was unexpected. The failure of renal denervation to alter NCC activity may reflect the role of norepinephrine in the promotion of sodium

reabsorption in the proximal tubule and the loop of Henle via increased sodium transporter activity and decreased glomerular filtration rate. It is possible that renal denervation results in decreased sodium reabsorption in the proximal nephron, resulting in an increase in sodium delivery to the distal nephron that can stimulate NCC activity. This effect could be further exacerbated by potential age-related increases in the activity of sodium transporters in the proximal tubule and loop of Henle, which have been summarized elsewhere (Frame & Wainford, 2018) but have not been linked to excess norepinephrine. It is worth noting that our previous studies indicate that norepinephrine alone does not alter NCC activity during normal salt intake and instead prevents NCC suppression only during high salt intake in young rats (see Chapter 3), while our renal denervation studies in aged animals were performed only during normal salt intake. This raises the possibility that while renal norepinephrine content is increased in aged hypertensive animals during normal salt intake, it may not be relevant to altered NCC regulation until a high salt diet is introduced. Further studies will be required to determine the mechanism and potential pathological role of increased NCC activity, as well as the role of norepinephrine in NCC regulation, in age-related hypertension and salt sensitivity of blood pressure.

While the contribution of impairments in the acute sympathoinhibitory renorenal reflex to the long-term dysregulation of renal sodium handling and blood pressure require further study, the current findings raise the possibility that a decrease in mechanosensitive afferent renal nerve-mediated sympathoinhibitory

reno-renal reflex function promotes sympathoexcitation – perhaps due in part to reduced signaling of sympathoinhibitory neurons in the PVN – driving sodium retention and the development of salt-sensitive hypertension. Further, the data also provide evidence suggesting that the renal sympathetic nerves plays a pathophysiological role in age-related hypertension and salt sensitivity that may reflect inappropriate norepinephrine-mediated activation of the NCC. These findings are particularly significant given lifelong exposure to excessive dietary intake in elderly patients and evidence suggesting a pivotal role of sodium retention in treatment-resistant hypertension (Powles et al., 2013; Mozaffarian et al., 2014; Williams et al., 2015). We speculate that therapies reducing sympathetic outflow and sodium retention, including renal denervation, may be particularly useful in older hypertensive patients.

**CHAPTER SIX: GNAI2 Polymorphic Variance Associates with Salt
Sensitivity of Blood Pressure in the Genetic Epidemiology Network of Salt
Sensitivity Study**

This section was previously published as: X. Zhang, **A. A. Frame**, J.S. Williams, and R. D. Wainford. GNAI2 polymorphic variance associates with salt sensitivity of blood pressure in the Genetic Epidemiology Network of Salt Sensitivity study. *Physiological Genomics* 50(9):724-725. PMID: 29906209 © 2018 The American Physiological Society. For the purposes of this dissertation, the introduction has been expanded to include a greater amount of relevant background information.

Abstract

Salt sensitivity of blood pressure increases hypertension risk and associated adverse cardiovascular outcomes. At present, there are no validated rapid tests or diagnostic markers to identify salt sensitivity of blood pressure in clinical practice. Based on our prior animal studies that report a role for brain Gα_{i2} proteins in the salt sensitivity of blood pressure and evidence that GNAI2 single nucleotide polymorphisms (SNPs) associate with hypertension risk, we investigated the hypothesis that GNAI2 SNPs associate with salt sensitivity of blood pressure in humans. Our data provide the first evidence that a GNAI2 SNP (rs10510755) positively associates with salt sensitivity of blood pressure in the Genetic Epidemiology of Salt Sensitivity dataset (continuous phenotype P = 0.049, case-control phenotype P = 0.039; n = 968), independently of subject sex or age.

These observations suggest that genotyping at GNAI2 may be a useful biomarker in identifying individuals at risk for developing salt-sensitive blood pressure and related complications or in identifying salt sensitivity within the hypertensive population.

Introduction

Salt sensitivity of blood pressure, a clinical phenomenon characterized by an exaggerated pressor response to increased dietary sodium intake, is present in roughly 50% of hypertensive patients and 25% of normotensive individuals (Weinberger et al., 1986). Salt sensitivity promotes the development of hypertension and has been identified as an independent predictor of cardiovascular morbidity and mortality even in the absence of hypertension (Weinberger et al., 1986; Weinberger, 1996; Morimoto et al., 1997; Meneton et al., 2005; Appel et al., 2011). Despite the broad clinical and prognostic relevance of salt sensitivity, particularly given excessive global dietary sodium intake, tests of salt sensitivity are cumbersome, poorly standardized, and rarely used in the clinical setting. The identification of a biomarker of salt sensitivity would aid in cardiovascular risk stratification and allow for better targeted recommendations for preventive and therapeutic interventions, including reduced dietary sodium intake. Further, such a biomarker would enable the pursuit of mechanistic studies and the assessment of anti-hypertensive drug efficacy in defined salt-sensitive versus salt-resistant patients, which has been hampered by the cumbersome dietary

interventions currently used to test for salt sensitivity and by the somewhat arbitrary and unstandardized blood pressure cutoffs used to interpret such tests.

Salt resistance is characterized by dietary sodium-evoked sympathoinhibition, which facilitates natriuresis and the maintenance of normotension (Lohmeier et al., 1999; Brooks et al., 2005; Gao et al., 2005; Osborn et al., 2008; Johns et al., 2011; Johns, 2014). In contrast, sympathoexcitation promotes sodium retention and increased blood pressure in salt sensitivity (Bayorh et al., 1998; Miyajima & Yamada, 1999; Huang et al., 2001; Dobesova et al., 2002; Brooks et al., 2005; Yatabe et al., 2010; Stocker et al., 2013; Johns, 2014). A number of studies have demonstrated that the paraventricular nucleus (PVN) of the hypothalamus, one of several central regulatory sites that modulate sympathetic tone, sodium homeostasis, and blood pressure, plays a role in these responses (Budzikowski et al., 1998; Huang & Leenen, 1998; Huang et al., 1998; Huang et al., 2001; Akine et al., 2003; Badoer et al., 2003; Dampney et al., 2005; O'Donoghue & Brooks, 2006; Gabor & Leenen, 2009; Frithiof et al., 2014; Holbein & Toney, 2015; Larson et al., 2015). Further, central GPCR-coupled Gai₂ subunit proteins are required for appropriate activation of parvocellular neurons in the PVN, sympathoinhibition, and normotension in response to acute volume expansion and hypertonic saline infusion (Kapusta et al., 2012; Wainford et al., 2013; Carmichael et al., 2016). Central GPCR-coupled Gai₂ subunit proteins are also required for sympathoinhibition and the maintenance of salt resistance during high salt intake in Sprague Dawley and Dahl Salt Resistant rats, which exhibit

dietary sodium-evoked upregulation of Gai₂ protein expression specifically in the PVN (Kapusta et al., 2012; Kapusta et al., 2013; Wainford et al., 2013; Wainford et al., 2015; Carmichael et al., 2016). In contrast, Dahl Salt Sensitive rats fail to increase PVN Gai₂ subunit protein expression during high salt intake (Wainford et al., 2015). These findings suggest that central Gai₂ proteins are required for the homeostatic responses to sodium challenge that characterize salt resistance. Importantly, although variance in the GNAI2 gene encoding Gai₂ proteins has not been investigated in defined salt-resistant versus salt-sensitive populations, two single nucleotide polymorphisms in the GNAI2 gene have been linked to hypertension risk (Menzaghi et al., 2006; Kohara et al., 2008). Interestingly, one of these polymorphisms is a functionally relevant mutation in the GNAI2 promoter region, that results in decreased transcription (Menzaghi et al., 2006) – which could promote salt sensitivity by preventing dietary sodium-evoked upregulation of Gai₂ proteins.

In the current studies, we hypothesized that GNAI2 genetic variance would be associated with the salt sensitivity of blood pressure. To investigate this hypothesis, we analyzed genetic and phenotypic data procured from the Genetic Epidemiology of Salt Sensitivity (GenSalt) dataset to determine whether GNAI2 variance correlates with 1) the blood pressure response to increased dietary sodium intake as a continuous phenotype, and 2) the salt sensitivity of blood pressure as a binary trait.

Methods

Phenotype

In this analysis, salt sensitivity of blood pressure was defined as the change in systolic blood pressure observed between a 7-day restricted-sodium feeding (51.3 mmol/day) and a 7-day high-sodium feeding (307.8 mmol/day). As per GenSalt program design as previously reported (Group, 2007), all blood pressure readings were measured in the morning by trained and certified observers using a random-zero sphygmomanometer after 5 minutes of rest with the participant in the sitting position and the arm placed at the level of the heart. The blood pressure levels during the dietary sodium intervention were calculated as the mean of nine measurements obtained from three clinical visits on days 5, 6, and 7 of each dietary sodium intervention phase (Group, 2007). Salt sensitivity of blood pressure was defined by the observation of a greater than a 5mmHg (4.6-4.9mmHg rounded to 5mmHg) or greater increase in mean systolic blood pressure following the transition from a restricted to high-sodium intake (i.e., blood pressure response to high-sodium diet = blood pressure on high-sodium diet minus blood pressure on restricted-sodium diet).

Cohort details

The GenSalt population cohort study has been extensively defined previously. In brief, the GenSalt study was conducted in a Han Chinese population in rural north China during 2003–05. The study was approved by a Human

Research Oversight committee, and written consent was obtained from each participant. This population had a mean systolic blood pressure that ranged from 130-160mmHg without use of antihypertensive medications. All subjects were between 18-60 years, with a mean age of 38.5 ± 6 years (Group, 2007). A total of 1000 subjects in this study underwent whole genome genotyping using the Affymetrix Genome Wide Human SNP Array AFFY_6.0. Of this population, 968 had complete genotype and phenotype data available for analysis. Among these 968 subjects, we categorized 369 as “salt-sensitive” (175 females, 194 males) and 642 as “non-salt-sensitive” (330 females, 269 males). For the salt-sensitive subjects, the mean \pm SD of blood pressure response to high-sodium diet is 7.82 ± 3.20 . For the non-salt-sensitive subjects, the mean \pm SD of blood pressure response to high-sodium diet is -0.05 ± 3.16 .

Type of Study

Candidate gene.

Details of the single nucleotide polymorphisms studied

The candidate gene under investigation was GNAI2. Primary single nucleotide polymorphisms (SNPs) investigated were rs10510755, rs9852677, rs2282751, rs4547694, and rs2298952 identified from HapMap and 1000Genomes projects to capture 100% of the genetic variation in GNAI2.

Analysis Model

For the association analysis, the continuous phenotype was the difference in mean systolic blood pressure from a low- to a high-salt intake, which represents the quantitative trait of the salt sensitivity of blood pressure (i.e., blood pressure response to high sodium diet = blood pressure on high-sodium diet minus blood pressure on restricted-sodium diet). Linear regression was used to model salt sensitivity of blood pressure and an additive genetic model (SNP dosage) adjusted for age, sex, and principal components. In addition, we conducted a logistic regression analysis, which provides an odds ratio estimate, by treating the binary trait (salt-sensitive vs. non-salt-sensitive) as the outcome; SNP as predictor; and age, sex, and principal components as covariates. For all analyses, the significance threshold was considered at $P < 0.05$.

Results

Of the 5 GNAI2 SNPs selected for testing, only SNPs rs1010755, rs9852677, and rs2282751 were present in GenSalt after quality control. As such, only these 3 SNPs underwent analysis. SNPs rs9852677 and rs2282751 were not significantly associated with the salt sensitivity of blood pressure in the GenSalt dataset (Table 6.1). However, rs10510755 was associated with salt sensitivity of blood pressure (Table 6.1). For SNP rs10510755, the minor allele frequency (MAF) was 6.2% in the GenSalt population. The GenSalt dataset contains 369 salt-sensitive individuals in which SNP rs10510755 was identified in 118 independent

subjects. The minor allele at rs10510755 was more frequently observed within the salt-sensitive cohort compared to the nonsalt-sensitive cohort (salt-sensitive cohort 32% vs. non-salt-sensitive cohort 12%, $p < 0.05$) even after adjusting for age and gender. There was no additive SNP effect observed, however; only one subject exhibited two minor alleles in this study. SNP rs10510755 is associated with greater odds of the salt sensitivity of blood pressure (case-control phenotype odds ratio 3.046; 95% confidence intervals 2.195–4.228, Z-score 6.663, $P = 0.039$). This suggests that subjects with GNAI2 SNP rs10510755 are three times more likely to be salt-sensitive than subjects that lack SNP rs10510755.

<i>Linear-regression model (continuous phenotype)</i>											
rsID	Locus name	Chr	Position	EA/ALT	Trait	EAF	Effect-size (β)	Std. Error	Z-score	P	N
rs10510755	GNAI2	3	50,256,172	T/C	BP response to high sodium diet	0.0620	-0.4175	0.2175	-1.9192	4.90E-02	968
rs9852677	GNAI2	3	50,266,621	C/T	BP response to high sodium diet	0.4891	-0.1023	0.0955	-1.0716	2.80E-01	968
rs2282751	GNAI2	3	50,266,789	G/A	BP response to high sodium diet	0.4332	-0.0438	0.0966	-0.4530	6.50E-01	968
<i>Logistic-regression model (case-control phenotype)</i>											
rsID	Locus name	Chr	Position	EA/ALT	Trait	EAF	Effect-size (β)	Std. Error	Z-score	P	N
rs10510755	GNAI2	3	50,256,172	T/C	Salt Sensitive vs. non-Salt Sensitive	0.0620	-0.4420	0.2140	-2.0648	3.90E-02	369 (Yes), 599 (No)
rs9852677	GNAI2	3	50,266,621	C/T	Salt Sensitive vs. non-Salt Sensitive	0.4891	-0.0888	0.0950	-0.9353	3.50E-01	369 (Yes), 599 (No)
rs2282751	GNAI2	3	50,266,789	G/A	Salt Sensitive vs. non-Salt Sensitive	0.4332	-0.0381	0.0961	-0.3964	6.92E-01	369 (Yes), 599 (No)
<i>Logistic-regression model (case-control phenotype)</i>											
rsID	Locus name	Chr	Position	EA/ALT		EAF	Odds Ratio	95% CI	Z-score	P	N
rs10510755	GNAI2	3	50,256,172	T/C	Odds Ratio	0.0620	3.046	2.195 - 4.228	6.663	3.90E-02	369 (Yes), 599 (No)

Table 5.1 GNAI2 SNP results Abbreviations and definitions: rsID - dbSNP rsID; locus name, variant annotation; Chr - chromosome; Position - Build 37 position; EA/ALT - Effect allele and alternative allele; Trait; EAF - Effect allele frequency; β (SE)/Z-score - effect estimate and standard error for a quantitative trait and Z-score for example for a binary trait; CI - Confidence Interval for Odds Ratio; P - P-value for association; N - number of samples analysed.

Discussion

These results suggest a positive association between the GNAI2 SNP rs10510755 and salt sensitivity of blood pressure and provide important clinical relevance to our prior mechanistic work in animal models regarding the influence

of Gai2 proteins on the salt-sensitivity of blood pressure (Wainford et al., 2015). Possible limitations of the current analysis are the relatively small sample size, the lack of ability to include analysis of body mass index as a covariate, and the low minor allele frequency of the indexed SNP. These data suggest GNAI2 polymorphic variance represents a potential biomarker for salt sensitivity of blood pressure that may identify a specific subset ($\approx 32\%$ based on GenSalt dataset) of salt-sensitive subjects.

CHAPTER SEVEN: Overall Discussion

Summary

The current studies were designed to investigate the integrated renal and neural mechanisms that contribute to sodium homeostasis and blood pressure regulation, with the goal of identifying potential mechanisms and therapeutic targets in the pathophysiology of salt-sensitive hypertension. We first investigated the renal and sympathetic nervous system mechanisms determining salt resistance versus salt sensitivity in male young adult Sprague Dawley rats, which are classically salt-resistant but become salt-sensitive during norepinephrine infusion (Walsh et al., 2016), and extended some of our key findings in Dahl Salt Resistant and Dahl Salt Sensitive rats. Based on evidence that human aging is associated with increases in the prevalence of both hypertension and salt sensitivity as well as elevated sympathetic tone (Luft et al., 1987; Luft et al., 1991; Esler et al., 1995; Esler et al., 2002; Muntner et al., 2018; Whelton et al., 2018), we then extended our studies using the male Sprague Dawley rat as a model of normal aging.

In Chapter 3, we identified an α_1 -adrenoceptor-gated WNK1/OxSR1 signaling pathway that prevents the suppression of NCC-mediated sodium reabsorption during high salt intake, promoting the development and maintenance of salt-sensitive hypertension in norepinephrine-infused young adult Sprague Dawley rats. In Chapter 4, we described a critical role for the mechanosensitive afferent renal nerve-mediated sympathoinhibitory reno-renal reflex in the

maintenance of salt resistance in young adult Sprague Dawley rats and provided novel evidence implicating the PVN in the central integration of the afferent and efferent arms of the sympathoinhibitory reno-renal reflex. In Chapter 5, we extended these findings using the Sprague Dawley rat as a model of normal aging and demonstrated that aging animals develop salt-sensitive hypertension driven in part by significant and progressive impairment in two mechanisms that contribute to salt resistance in young animals – namely, the suppression of sympathetic outflow and NCC activity that promotes natriuresis during high salt intake and the mechanosensitive afferent renal nerve-mediated sympathoinhibitory reno-renal reflex responses that promote natriuresis during acute sodium challenge in young rats. In Chapter 6, we demonstrated that a single nucleotide polymorphism in the *GNAI2* gene encoding $G\alpha_{i2}$ proteins is associated with salt sensitivity of blood pressure in the GenSalt cohort, providing translational relevance to prior studies indicating that $G\alpha_{i2}$ proteins in the PVN may be required for salt resistance in animal models.

Future Directions

Interrogation of the α_1 -Adrenoceptor-Gated WNK1/OxSR1 NCC Regulatory

Pathway Using Lentiviral Knockdown

Our studies of the adrenergic regulation of NCC activity in young animals in Chapter 3 revealed an α_1 -adrenoceptor-gated WNK1/OxSR1 signaling pathway that provides a number of potential therapeutic targets in salt-sensitive

hypertension. The utility of these targets in reducing NCC activity and attenuating blood pressure in norepinephrine-evoked salt-sensitive hypertension and age-related hypertension and salt sensitivity could be assessed via the targeted lentiviral knockdown of each kinase in vivo. These experiments would also provide mechanistic insight to strengthen our observational studies. Further, infusion of norepinephrine directly into the renal artery would allow for the assessment of the specific role of renal adrenergic signaling in the regulation of NCC activity, sodium homeostasis, and blood pressure, refining the current studies performed using systemic norepinephrine infusion.

Assessment of NCC Post-Translational Modification and Localization

While our studies in Chapter 3 indicate that the failure to suppress NCC activity during high salt intake in young norepinephrine-infused rats could be due in part to increased NCC expression and phosphorylation at Thr58, it is possible that other alterations known to impact NCC activity, including phosphorylation at other sites, ubiquitinylation, or alterations in NCC trafficking to the plasma membrane, could also contribute to NCC dysregulation (Moriguchi et al., 2005; Sandberg et al., 2006; Richardson et al., 2008; Arroyo et al., 2011). The unbiased assessment of post-translational modifications using mass spectrometry, as well as the precise cellular localization of the NCC under different dietary conditions using electron microscopy, would yield important insight into the regulation of NCC activity. These studies could be particularly clarifying in aged rats, which exhibit an

increase in physiological NCC activity in the absence of any change in total NCC expression and an unexpected decrease in phosphorylation at Thr53.

Investigation of the Relationship between the Renal Nerves and the NCC

Based on our observations that norepinephrine infusion evokes salt-sensitive hypertension characterized by failure to suppress NCC activity in young, classically salt-resistant rats, we anticipated that removal of the renal sympathetic nerves would reduce NCC activity and blood pressure in aged hypertensive animals that exhibit increased renal sympathetic tone. However, our studies in chapter 5 demonstrated that selective afferent renal nerve ablation and bilateral renal denervation, which removes both the afferent renal nerves and the renal sympathetic nerves, improved blood pressure but did not attenuate NCC activity in hypertensive aged Sprague Dawley rats. It is important to note that these studies were performed during normal salt intake, while our studies suggest that 1) norepinephrine has no impact on NCC activity during normal salt intake but prevents NCC suppression during chronic high salt intake in young adult rats (Walsh et al., 2016), and 2) aged animals exhibit a failure to suppress renal sympathetic tone and NCC activity during high salt intake. Denervation studies in aged animals during high salt intake will therefore provide important insight into the potential role of the renal nerves in NCC regulation during acute and chronic sodium challenge.

Further, radiotelemetry studies in which renal denervation is performed subsequent to the establishment of chronic NCC antagonism in aged hypertensive rats will yield interesting insight into the relationship between the renal nerves and the NCC. It is possible that the efficacy of renal denervation is masked by the use of thiazide diuretics. Conversely, it is possible that renal denervation during thiazide treatment results in a synergistic effect, producing a larger decrease in blood pressure than renal denervation or NCC antagonism alone. The findings from these studies will be particularly relevant to the interpretation of clinical trials in which renal denervation is often performed in subjects in whom existing anti-hypertensive therapies are maintained.

Investigation of Other Renal Sodium Transporters

In Chapter 3, we elected to focus on the NCC based on previous studies indicating a specific role for impaired regulation of NCC activity in the development of norepinephrine-evoked salt-sensitive hypertension (Mu et al., 2011; Uchida et al., 2012; Terker et al., 2014; Walsh et al., 2016). Because aging is associated with increases in both salt sensitivity and sympathetic tone (Luft et al., 1987; Luft et al., 1991; Esler et al., 1995; Esler et al., 2002), we maintained our focus on the NCC in the renal sodium transporter studies in Chapter 5. Interestingly, while increased NKCC2 and ENaC activity have been linked with salt-sensitive hypertension in young animal models (Husted et al., 1996; Alvarez-Guerra & Garay, 2002; Aoi et al., 2007; Amin et al., 2011; Haque et al., 2011), aging is associated with

decreased NKCC2 and ENaC expression and activity in several animal models (Tian et al., 2006; Tiwari et al., 2009). While our studies indicate that increased NCC activity promotes age-related hypertension, the role of other transporters in age-related hypertension and salt sensitivity of blood pressure has not been fully elucidated and warrants further study.

Characterization of Age-Related Impairments in the Afferent Renal Nerves

Our studies in Chapters 4 and 5 suggest that the mechanosensitive afferent renal nerves are required for the responses to acute and chronic sodium challenge in young rats, and that age-related hypertension is characterized by impairments in the afferent renal nerve-mediated reno-renal reflex. However, the nature of this impairment requires further investigation. Together with our acute in vivo sodium challenges, our ex vivo studies of afferent renal nerve function suggest that there is a primary issue with the afferent renal nerves. While the nature of age-related changes in the afferent renal nerves has not been investigated, declines in other sensory systems have been attributed to reduced density, atrophy, and impaired electrophysiological function of sensory afferent nerve fibers in elderly humans and animal models of aging. Future studies will investigate whether the observed impairment in afferent renal nerve responsiveness reflects the physical loss of afferent renal nerve fibers, impairments in the signaling pathways involved in afferent renal nerve activation, or altered electrophysiological afferent renal nerve function. Further, it is possible that the structural and mechanical properties of the

renal pelvis, such as elasticity, could change with age, influencing the degree of mechanical stress activating afferent renal nerve mechanoreceptors during a given stimulus. Future studies in aged animals replicating those performed in young adult Sprague Dawley rats in Chapter 4, in which 1) renal pelvic pressure was measured during acute volume expansion in anesthetized animals, and 2) the natriuretic responses to direct in vivo manipulation of renal pelvic pressure and sodium concentration were assessed, will help to elucidate the mechanistic underpinnings of age-related afferent renal nerve dysfunction.

*Functional Assessment of the Parvocellular PVN Neurons Activated by Acute
Volume Expansion*

In Chapter 4, we provided evidence that the afferent renal nerves are required for the activation of sympathoinhibitory neurons in the PVN during acute volume expansion. It is therefore likely that the reduction in volume expansion-evoked Fos activation in the PVN of aged rats reflects the observed reduction in afferent renal nerve responsiveness. However, it is possible that aging is also associated with impairments downstream of afferent renal nerve activation, including altered signal transduction within the PVN itself, which could contribute to age-related alterations in renal sodium handling and blood pressure regulation.

Our assessment of neuronal activation in the PVN is based on Fos expression. Although our findings are supported by previous studies demonstrating that a volume expansion activates sympathoinhibitory neurons in

the PVN (Haselton et al., 1994; Patel & Zhang, 1994; Randolph et al., 1998; Cunningham et al., 2002; Howe et al., 2004) and results in the suppression of renal sympathetic outflow (Haselton et al., 1994; Kapusta et al., 2012), further evidence is needed to validate that the parvocellular PVN neurons that exhibit Fos induction following volume expansion are 1) functionally activated, 2) sympathoinhibitory in nature, and 3) involved in the natriuretic responses to acute sodium challenge. The functional nature of the age-related reduction in Fos expression in parvocellular PVN neurons should also be confirmed. These remaining questions could be addressed in part by studies of the impact of PVN lesion on the natriuretic response to acute volume expansion. Further, our findings would be strengthened by renal sympathetic nerve recordings that would confirm whether selective afferent renal nerve ablation, or PVN lesion, prevents the suppression of renal sympathetic outflow during volume expansion, and whether age-related impairments in afferent renal nerve function and PVN responses are linked with blunted renal sympathoinhibition during acute volume expansion.

Validation of Findings in Other Models of Aging and Human Hypertension

In Chapter 5, we provided evidence that age-related impairments in the afferent renal nerve-mediated sympathoinhibitory reflex and NCC regulation promote hypertension and salt sensitivity. While we designed our studies to temporally separate the development of salt-sensitive hypertension from the development of age-related renal damage, which can confound studies of the

intrinsic renal mechanisms driving hypertension, it is important to note that the translation of animal age to human age is difficult and varies by the physiological system under investigation. Our findings must be validated in other animal models of aging and, ideally, investigated in human subjects or perhaps initially in tissue obtained from human subjects. Critically, many animal studies that have investigated age-related alterations in renal sodium handling have not directly assessed blood pressure or salt sensitivity, raising the possibility that seemingly conflicting evidence in the literature regarding sodium transport may be easily resolved with insight into blood pressure phenotype. For instance, evidence that aging Fischer Brown Norway rats exhibit increase baseline NCC expression (Tian et al., 2006) seems to conflict with the observation that aging C57Bl6/CBA/129 mice exhibit decreased baseline NCC activity (Tiwari et al., 2009). However, the decrease in baseline NCC activity in aged mice could play a protective role, as the same study observed that these animals do not exhibit an age-related increase in blood pressure (Tiwari et al., 2009). In contrast, while blood pressure was not measured in the study of Fischer Brown Norway rats, other studies have demonstrated that these animals develop age-related hypertension (Chugh et al., 2012; Chugh et al., 2013; Pokkunuri et al., 2015), and it is possible that the increase in NCC expression plays a causal role. Together with blood pressure data, these apparently conflicting findings support the unified theory that NCC regulation is linked closely to the regulation of blood pressure in aging animals.

Identification and Validation of Biomarkers of Salt Sensitivity

In Chapter 6, we demonstrated that polymorphic variance in GNAI2, the gene encoding Gα_{i2} proteins that are critical for the maintenance of salt resistance in young animals, associates with salt sensitivity of blood pressure in the GenSalt dataset. The GenSalt study, which yielded full genotypic and phenotypic data for 1000 individuals of Han Chinese descent, was relatively limited in both size and population diversity (Group, 2007). Thus, our findings must be replicated in a larger and more diverse population. It is also important to consider biomarkers that may be more broadly relevant in the absence of genetic variation. In Chapter 5, we observed that salt-sensitive hypertension in aged animals is associated with impaired afferent renal nerve responsiveness, assessed as substance P release, even during normal salt intake. These findings raise the possibility that urinary substance P, or perhaps other substances involved in afferent renal nerve activation including prostaglandin E₂ and calcitonin gene-related peptide (Kopp et al., 1997; Gontijo et al., 1999; Kopp et al., 2000), could serve as rapid, noninvasive biomarkers of salt sensitivity.

Significance and Perspectives

Hypertension affects one in two adults in the United States and contributes to 10% of global deaths. While there is a demonstrated benefit of blood pressure reduction on measures of hypertension-related morbidity and mortality, less than half of hypertensive patients achieve therapeutic blood pressure control. These

studies sought to delineate the integrated renal and neural mechanisms that modulate sodium homeostasis and blood pressure regulation in the setting of salt sensitivity, which promotes hypertension, and aging, which is associated with both hypertension and salt sensitivity. The goal of these studies was to identify novel therapeutic targets and inform current treatment paradigms for hypertension, meeting an urgent public health need.

Overall, our findings have important implications for the application of therapies targeting the sympathetic nervous system and the NCC. The observation that NCC antagonism reduces blood pressure in age-related hypertension was not unexpected, in light of its use as a first-line anti-hypertensive therapeutic – but is informative given a recent report that thiazides are currently under-prescribed, particularly in the aging population. Further, although α_1 -adrenoceptor blockade is not a first-line anti-hypertensive therapeutic and poses several risks that are magnified in the aging population, including orthostatic hypotension, our findings suggest that renal α_1 -adrenoceptor signaling may be an important therapeutic target in salt-sensitive and age-related hypertension. Selective small-molecule WNK inhibitors have a beneficial effect on blood pressure in rodent models of human WNK overexpression, providing a potential avenue for the downstream modulation of renal α_1 -adrenoceptor signaling pathways that could avoid the adverse outcomes associated with systemic α_1 -adrenoceptor blockade.

Our findings may be particularly informative to the targeted application of renal denervation. Variability in afferent renal nerve activity has been proposed as

a contributing factor to the mixed results of renal denervation trials, and our findings raise the possibility that renal nerve ablation could disrupt a protective sympathoinhibitory reno-renal reflex in some individuals, blunting the efficacy of renal nerve ablation or even exacerbating hypertension. In contrast, individuals in whom the sympathoinhibitory reno-renal reflex is impaired may be ideal candidates for renal denervation. The development of biomarkers or simple non-invasive challenges designed to test the balance of sympathoinhibitory versus sympathoexcitatory reno-renal reflexes – for instance, intravenous volume expansion, which has been used in human studies of renal physiology for decades – and biomarkers of salt sensitivity, including GNAI2 polymorphic variance, could guide patient selection for phenotypically targeted renal nerve ablation.

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CURRICULUM VITAE

